UNIVERSITY OF ALBERTA

EFFECTS OF FORMULATION AND CHEMICAL MODIFICATION ON PEPTIDES: STABILITY IN HUMAN SERUM AND MOUSE SERUM.

By

ULUNMA OBIOMA IMO

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Dr. W. Dixon

Dr. J. Samuel

Dr. G. S. Kwon

Dr. F. M. Pasutto

Dr. D. Wishart

Date 23-1-98

TO MY BELOVED HUSBAND FERDINAND AND DAUGHTER LINDA.

Abstract

Synthetic peptides are vaccine candidates. However, they are poor immunogens *in vivo* due to their rapid degradation in biological fluids. Chemical modification and formulation of peptides may increase their stability *in vivo*. This study investigated the effects of chemical modification and formulation on the degradation kinetics of three synthetic peptides in human and mouse serum using reverse phase HPLC. A human MUC1 peptide and its multiple antigen peptide (MAP-4) analog (candidates for cancer vaccines), an ovalbumin peptide (Ova $_{323-339}$) and its biotin analog, another ovalbumin peptide (Ova $_{257-264}$) and its formulation in PLGA microspheres were studied. MAP clustering and biotinylation increased the serum half-lives of peptides by three and five fold respectively, while the formulation in PLGA microspheres increased the half-life by 132 fold. This study indicates that MAP clustering, biotinylation and formulation in PLGA microspheres will increase the peptide stability in biological fluids, thus increasing their immunological effectiveness.

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List of Abbreviations

AUFS	absorbance unit full scale
DDH_20	double distilled deionized water
DMSO	dimethyl sulfoxide
HPLC	high performance liquid chromatography
HS	human serum
MS	mouse serum
min.	minute(s)
MUC1	mucin1
PBS	phosphate buffered saline
PLGA	poly(d,1-lactic acid-co-glycolic acid)
PVA	polyvinyl alcohol
sec.	second(s)
TCA	trichloroacetic acid
UV	ultraviolet
VNTR	variable number tandem repeat

A. Introduction

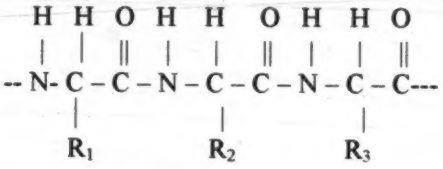
The vast majority of the currently used therapeutic substances are products of chemical synthesis. Until recently, the only biologically based therapeutic agents available have been blood products, vaccines and some enzymatic preparations. Such biological products have an associated risk of disease transmission due to pathogens derived from infected raw materials.[1]

Many therapeutic proteins destined for use in the health care industry have been developed only recently. A host of other proteins have, however, found wide industrial applications. The majority of such industrial proteins are enzymes that are normally obtained by fermentation from various microbial sources. Unlike proteins destined for therapeutic application, these enzymes are produced in bulk quantities.[1]

Since proteins possess chemical and physical properties which present unique difficulties in their purification, separation, storage and delivery, the formulation of proteins is thus substantially different from that of small organic molecules.[2] In light of this, it is important to study peptide instability and the effect of proteolytic enzymes with a view towards better understanding protein stability at the molecular level. This understanding will lead to the solution of some of the problems associated with peptide vaccine formulation. The general characteristics of peptides will now be discussed taking into consideration delivery problems and the effect of proteolytic enzymes.

1.1 General Characteristics of Peptides

Amino acids are the building blocks of both peptides and proteins. Amino acids have the general formula consisting of free amino and carboxyl groups attached to the α-carbon which also has attached to it a hydrogen atom or an organic substituent, the R group or side chain. An example of a peptide backbone is shown below.



Amino acid side chains vary considerably in their physical chemical properties. They are generally classified according to their polarity: some are non-polar (hydrophobic) while others are polar (hydrophilic). Peptides with both ionizable acidic and basic side chains have characteristic isoelectric points (pI) so the overall net charge and polarity of peptides in aqueous solution varies with pH. Hydrophobicity and hydrophilicity, together with the number of charged groups in a peptide, are important factors in the separation of peptides.[4] It is these chemical properties of peptides in addition to their physical properties that present difficulties in the purification, separation, and delivery of peptides.[2] Processes contributing to peptide instability include proteolysis,[5] deamidation,[6] oxidation,[7] racemization,[8] and β-elimination.[9]

Peptide degradation may occur during production, isolation, purification, analysis, delivery or storage[2] and because of the ubiquitous nature of these reactions, they are of tremendous concern to the pharmaceutical scientist.

In the last two decades, the number of biologically active peptides isolated and sequenced has increased dramatically. Through innovations in solid phase synthesis and rational drug design, chemists have been able to synthesize many peptides and peptide derivatives with novel therapeutic potentials. These have been used for a variety of physiological and pharmacological studies. Some of these synthetic peptides are now promising vaccine candidates for several viral, bacterial and parasitic diseases.[10] Although synthetic peptides offer several advantages (i.e. low cost, easy production and better definition of vaccine composition) over classical approaches to vaccine development, they have some limitations: the most well documented being their poor immunogenicity.[10]

Synthetic peptides have been used in several biological systems and as therapeutic agents either singly or in conjugation with other compounds. The pharmaceutical development of peptides is not without problems. As opposed to low molecular weight secondary metabolites / or proteins, many of the problems encountered with peptide pharmaceuticals are associated with their low stability.[11]

Mucins, such as the mucin MUC1 are extensively glycosylated high molecular weight (>200,00) proteins expressed by normal and malignant mammary epithelial cells.[12] In breast cancer the expression of MUC1 mucin is increased. Further, altered glycosylation in cancer exposes previously hidden MUC1 mucin epitopes to the immune system.[12] These peptides are the target for anti-MUC1 immune responses, which give a differential reaction in cancer compared with normal tissue.[12] The amino acid residues APDTR or

adjacent amino acid residues, have been reported to be highly immunogenic in mice for antibody production (after immunization with either breast cancer cells, human milk fat globule [HMFG] or the VNTR peptide).[12] In addition, human studies have shown that this region of MUC1 VNTR function as target epitopes for cytotoxic T cells.[13,14]

Small peptides generally are devoid of any three-dimensional structure in aqueous solution[15,16] and, as a result, are susceptible to enzymatic degradation in biologic fluids. This may restrict their use as biologic modifiers *in vivo*.[17] The breakdown of protein and peptide is generally initiated by endopeptidases (also known as proteinases).[18] These enzymes hydrolyze internal peptide bonds within the long polypeptide chains after which individual amino acid residues are removed consecutively by exopeptidases (peptidases) from the end of the fragments.[18] Much research has been conducted on these enzymes and the mechanism by which they degrade peptides.[15] A brief review of the characteristics of some of these enzymes follows.

1.2 Proteases

1.2.1 Biology of Proteases

Proteases, as previously stated, are enzymes that break down proteins into short chains of amino acids. They are divided into two groups: exopeptidases and endopeptidases.[19]
The term proteinase is applied only to proteases that exhibit endopeptidase activity while the term peptidase is applied to proteases that exhibit exopeptidase activity.
A protease, in short, is regarded as a proteinase if it exhibits a significant endopeptidase activity irrespective of whether it exhibits exopeptidase activity or not.[19] Enzymatic degradation of peptides by proteases may proceed in a stepwise fashion from either the

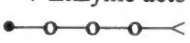
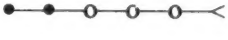
amino or carboxyl termini by exopeptidases, or internally by endopeptidases, with specificity being dependent on configuration, amino acid sequence and conformation.[20]

1.2.1.1 Exopeptidases

As stated earlier, exopeptidases degrade peptide chains from either the C or N terminal. They are classified into various groups according to their substrate specificity and named according to the site (terminus) of attack and the size of the liberated fragments.[19]

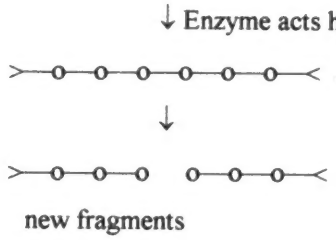
The table below shows the different classes of exopeptidases with illustrations indicating the site at which they attack peptide bonds.[19]

Table 1. Different classes of the exopeptidases.[19]

CLASS	SITE OF ACTION	ILLUSTRATION
Amino peptidase (aminoacyl or alpha(α)-aminoacylpeptide hydrolases)	α- peptide bond at the N-terminal	↓ Enzyme acts here 
Dipeptidyl peptidase (dipeptidyl peptide hydrolase)	β- peptide bond at the N-terminal	↓ Enzyme acts here
Tripeptidyl peptidase (tripeptidyl peptide hydrolase)	γ-peptide bond at the N-terminal	↓ Enzyme acts here 
Carboxpeptidase (peptidyl amino acid hydrolase)	α- peptide bond at the C- terminal	↓ Enzyme acts here
Peptidyl dipeptidase (peptidyl dipeptidase hydrolase)	β- peptide bond at the C-terminal	↓ Enzyme acts here
Dipeptidase	Peptide bond between two amino acids	↓Enzyme acts here
Tripeptidase (tripeptide aminopeptidase)	α- peptide bond at either the N- or C-terminal	↓Enzyme acts here OR ↓Enzyme acts here
Omega peptidase	Peptide bond of peptide that lack a free α-amino or α-carboxyl group or are linked through a scissile bond that involves a carboxyl or amino group which is not attached to a carbon atom.	↓Enzyme acts here OR ↓Enzyme acts here ◆ refers to modified or derivatized amino acid

1.2.1.2 Endopeptidases

Unlike exopeptidases, endopeptidases degrade peptide chains in the middle of the peptide sequence as shown below. They are classified into various groups based on their mechanism of action.[19]



Four main classes of endopeptidases are known: serine proteinases, cysteine proteinases, aspartic proteinases and metallo proteinases.

In general, the catalytic site in endopeptidases lies in the cleft on the surface of the enzyme molecules.[21] The substrate polypeptide binds to the active site of the cleft with the scissile peptide bond adjacent to the catalytic residues.[21] The amino acid residues of the substrate are designated P1, P2, P1′, P2′ and the corresponding subsites of the enzyme S1, S2, S1′, S2′. The S1 and S2 subsites are on the carbonyl side of the scissile peptide bond toward the N - terminus of the substrate, and the S1′, S2′ subsites are distal from the scissile bond toward the C - terminus of the substrate.[22] The subsites on the sides of the catalytic site interact with individual amino acid residue side chains of the polypeptide.[22]

The catalytic residues of serine proteinases are referred to as the catalytic triad - $Ser._{195}$, $His._{57}$ and $Asp._{102}$, based on the chymotrypsin numbering system.[21]These residues form a hydrogen bonding system. The reactive gamma (γ) - hydroxyl group of the $Ser._{195}$ attacks the carbonyl group of the scissile amide bond of the substrate to form a tetrahedral

intermediate. Decomposition of this intermediate results in the release of the amino portion of the substrate and the formation of an acyl enzyme. Subsequent hydrolysis of the acyl enzyme to an active enzyme also occurs via another tetrahedral intermediate.[23] During this process a carboxylic acid is produced.[23]

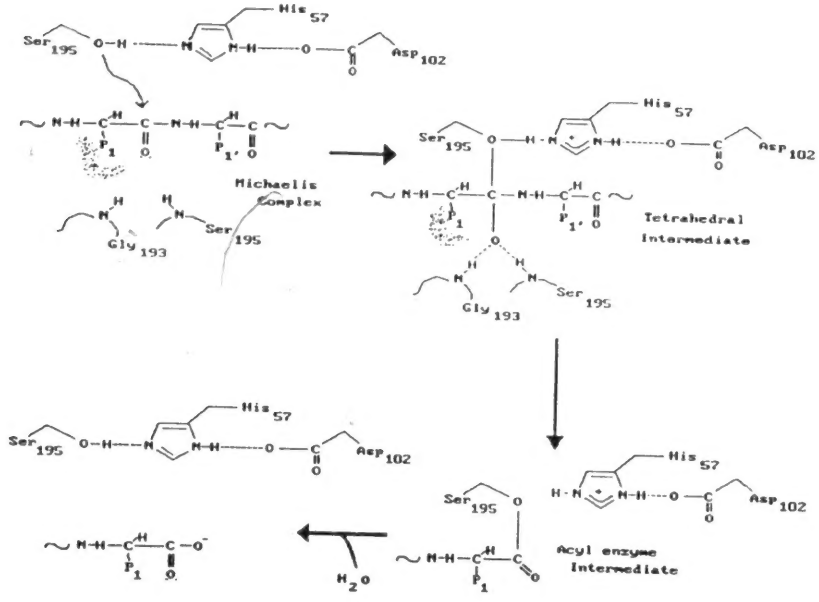


Figure 1. Schematic representation of the steps involved in catalysis by serine proteinases.

In cysteine proteinases the active enzyme contains a catalytic cysteine sulfhydryl group and a histidine imidazole group. It is the sulfhydryl group that attacks the amide or ester carbonyl group of a substrate to form a thioester covalent intermediate. The acyl group of the thioester is then hydrolyzed resulting in the release of the carboxylic acid portion of the substrate (not shown). An active enzyme is regenerated. The imidazole group of histidine, which acts as a proton donor or acceptor in the process,[23] catalyzes this step.

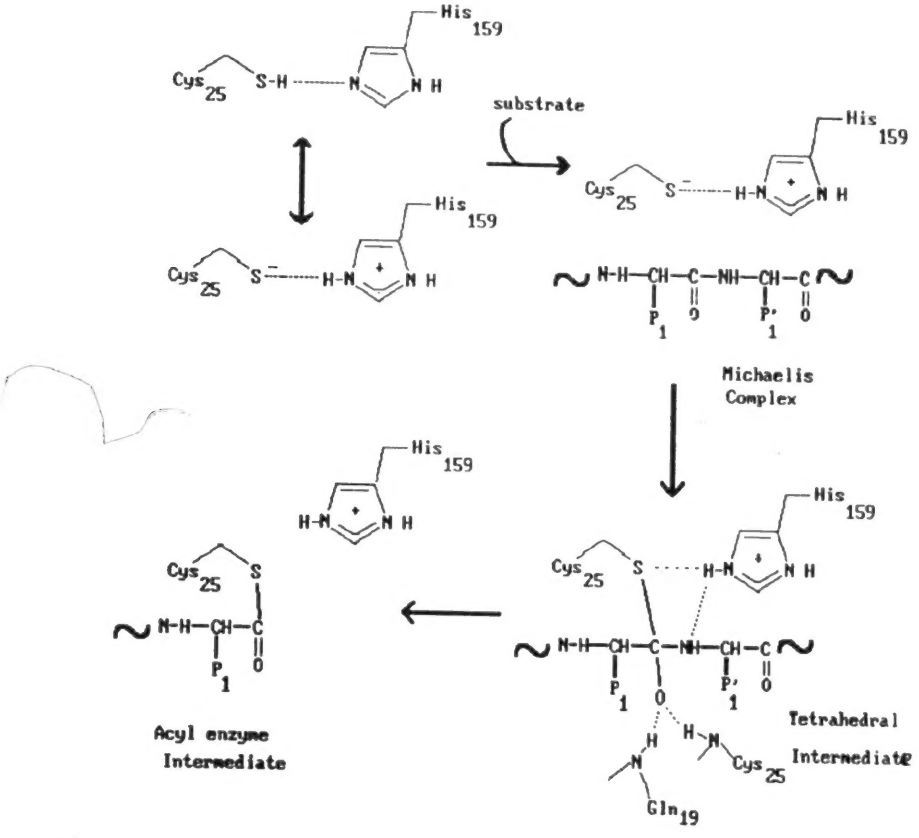


Figure 2. Schematic representation of the steps involved in catalysis by the cysteine Proteinases.

The active site in aspartic proteinases incorporates two active site aspartic acid residues (Asp_{-32} and Asp_{-215}, of the porcine pepsin numbering system). [24] Cleavage commonly involves an acid - base mechanism in which water attacks the carbonyl of the scissile peptide bond. The carboxylates at the active site mediate the appropriate proton transfer to form a tetrahedral intermediate.[25] Breakdown of the tetrahedral intermediate gives product complex containing both halves of the substrate, and the scheme below shows that dissociation of either an follow to give acyl product or amino product complex.[23]

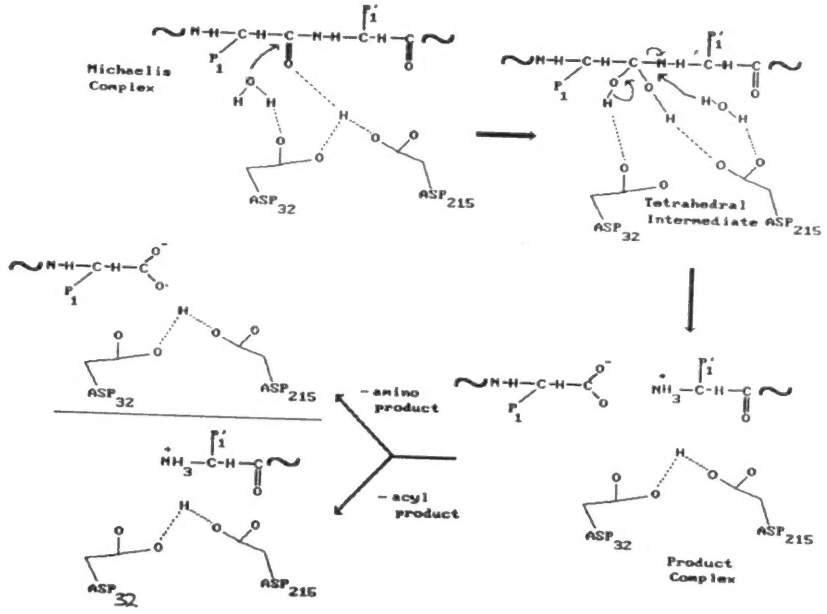


Figure 3. Schematic representation of the general acid-general base catalytic mechanism for the aspartic proteinase.

Metallo proteinases consist of a zinc atom and four ligands, two histidines, a glutamic acid residue and a water molecule, arranged in a terahedral geometry.[26] The substrate binds to the active site with the scissile peptide carbonyl group coordinating to the active site of the zinc. The zinc polarizes the scissile peptide carbonyl group and an adjacent glutamic acid residue acts as a general base catalyzing the addition of water to the substrate.[21] A schematic representation[23] of this process is shown below.

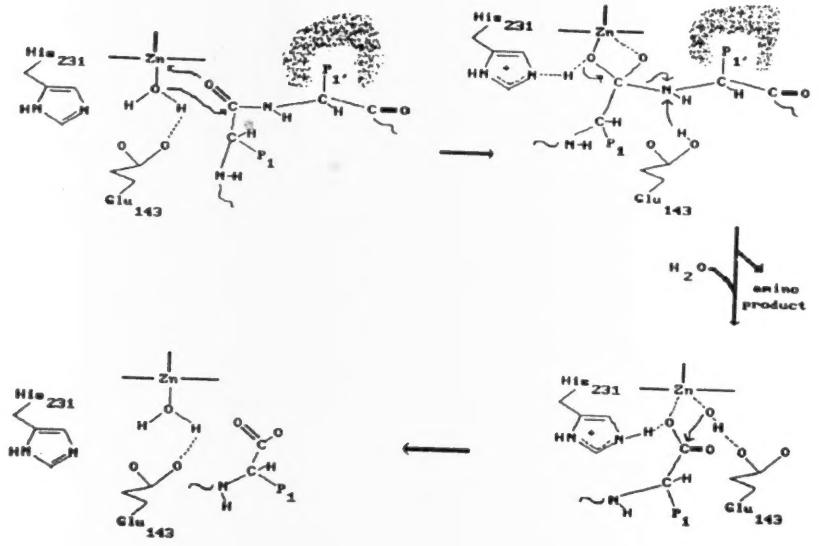
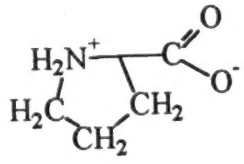


Figure 4. Schematic representation of the catalytic mechanism of the metallo proteinase.

1.2.2 Effect of Amino Acid Residues on Peptide Stability

The side chain each amino acid residue in proteins gives it its unique physical and chemical structure. In the imino acid proline, the nitrogen of the α - amino group has one hydrogen atom less and is bonded to the third carbon of the R group forming a cyclic ring as shown below. This structural difference from the other 19 naturally occurring amino acids affects the overall polarity of the proline residue.[27] The inclusion of a proline residue in the sequence of a peptide has been reported to result in unique conformational preferences that may or may not be of biological significance.[27] Collagen, a structural protein and hemoglobin, a transport protein are examples of proteins that have large quantities of the imino acid proline.[3] Because of the significant characteristics proline confers to the stability of peptides, it will be discussed in greater detail.

Proline has the general structure illustrated below.



Peptide bonds involving proline residues are often resistant to the actions of peptidases, sometimes even to peptidases known to have a wide range specificity.[28] In order to successfully initiate or complete the degradation of peptides and proteins to their constituent amino acid, peptidases, which can uniquely recognize the pyrrolidine ring of proline must be present.[28] Proline-specific peptidases are also restricted in their action depending on the orientation or position of proline in a peptide or substrate.[28]

There are four kinds of proline specific peptidases known to date: 1) proline specific endopeptidases, 2) proline specific N-terminal exopeptidases, 3) proline specific C-terminal exopeptidases, and 4) proline specific dipeptidases.

Proline specific endopeptidases are known to hydrolyze substrates in the carboxyl portion of proline residues, usually located internally in a peptide or ester, [28] as illustrated below.

↓Enzyme acts here

Y.....Pro....Y′

↓

Y.....Pro Y′

Y: peptide or protected amino acid

Y′: amino acid, peptides, amides, aromatic amines or alcohol[28]

Proline specific N-terminal exopeptidases are divided into three groups: post-proline dipeptidyl aminopeptidase, proline iminopeptidase and aminopeptidase.[28]

(i) The post-proline dipeptidyl aminopeptidase, also known as dipeptidyl aminopeptidase IV, hydrolyzes a peptide bond in the carboxyl portion of proline when proline is the penultimate amino acid of the free amino terminus. This hydrolysis results in the release of an X-pro dipeptide, [28] as illustrated (overleaf);

↓Enzyme acts here

X....Pro....Y

↓

X....Pro Y

X: amino acid

Y: amino acid, peptide , aromatic amine or alcohol

(ii) Proline iminopeptidase, also known as cytosol aminopeptidase, is an aminopeptidase which catalyzes the release of an N-terminal proline residue from peptides of any length.[26]

↓Enzyme acts here

Pro....Y

↓

Pro Y

Y: amino acid or peptide of variable length

(iii) Aminopeptidase P, also known as X - prolyl aminopeptidase, is an exopeptidase which cleaves the bond between any N - terminal amino acid and a penultimate proline residue.[28]

↓Enzyme acts here

X....Pro....Y

↓

X Pro....Y

X: amino acid

Y: amino acid, peptide or - OH[29]

Proline specific C - terminal exopeptidases including prolylcarboxypeptidase and carboxypeptidase P, catalyze the release of an amino acid residue from the C - terminus of a peptide provided that the penultimate residue is proline.[30]

↓Enzyme acts here

Y....Pro....X

↓

Y....Pro X

Y: protecting group or peptide

X: amino acid

Proline specific dipeptidases are divided into two groups: the prolyl dipeptidase and the proline dipeptidase.[28]

(i) The prolyl dipeptidase, also known as prolinase or iminodipeptidase shows high substrate specificity for dipeptides containing an N-terminal prolyl or hydroxyprolyl residues.[31,32] An illustration of the catalytic reaction for the prolyl dipeptidase is shown below:

↓Enzyme acts here

Pro....X

↓

Pro X

X: amino acid

(ii) Proline dipeptidase, also known as prolidase or imidopeptidase hydrolyses dipeptides in which the C - terminal residue is a proline.[31]

↓Enzyme acts here

X.... Pro

↓

X Pro

X: amino acid

1.2.3 Proteases in Human Serum

The total blood volume in the vascular system is approximately 8% of the body weight, corresponding to about 5 or 6L in an adult.[33] The fluid portion of the blood is called the plasma, and serum is the clear pale yellow liquid that remains after removal of cells and the clotting components from blood (fibrinogen, the precusor of fibrin). Thus it is similar to the plasma except for the absence of the clotting factors and white blood cells.[33] The solutes of the blood plasma make up 10% of the volume; proteins: albumin, globulins and fibrinogen accounting for about 7% of the volume; inorganic salts such as bicarbonate, chloride, phosphate and others accounting for 0.9% of the volume; the remaining percentage is made up of non-protein organic constituents like urea, amino acids, bilirubin, creatine, creatinine, carbohydrates and a host of other small organic molecules.[33] A variety of proteases are also present in the human serum, including amino peptidases, carboxypeptidases and dipeptidases being the most common exopeptidases. The endopeptidases like serine and aspartic proteinases are also present in human serum.[18,19] These proteases have been discussed in great detail in sections 1.2.1.1 and 1.2.1.2.

1.2.4 Modification of Peptides

The major obstacles to the application of peptides as clinically useful drugs or vaccines include their poor biomembrane penetration, rapid enzymatic degradation and short biological half-life.[34,35] Several approaches have been used to modify peptides to increase their stability and to decrease potential problems associated with delivery.[36] Some of the approaches being investigated today include derivatization of peptides to produce prodrugs,[36] substitution of some amino acids at distinct positions within the peptide

sequence,[37] conjugation of peptide to protein carriers,[38] and multiple antigen peptides (MAP).[39] A brief discussion of these methods follows.

One possible approach to decreasing delivery problems associated with peptides has been to either derivatize the peptides to form pro-drugs or "transport forms" that are more lipophillic than the parent peptides.[36] This method has been reported to protect the peptide against enzymatic degradation, particularly in the blood or mucosal barrier.[36]

Experiments have demonstrated that single amino acid substitutions and glycosylation provides a powerful tool for optimizing the stability of peptides.[37] For example, an N-acetylglucosamine (Glc NAC) moiety has been shown to inhibit peptide degradation in human serum.[37] Another approach has found peptides protected by D-amino acids at both the N and C-terminal to be more stable than predicted, based on additions of single substitutions.[37]

Today it is possible to conjugate synthetic peptide antigens to protein carriers in an attempt to enhance the low immunogenicity of free peptides.[38] This has been carried out using several methods, which although they have resulted in site-specific antibodies, [38] are still limited by the immune response they elicit especially when used as vaccines.[39]

The MAP systems for peptide-based vaccines were designed to overcome limitations associated with conjugation to carrier proteins and to reduce stability problems.[39] The MAP system will be discussed in great detail later on.

1.3 Vaccines

Vaccines are preparations of antigenic components consisting of, derived from, or related to pathogens. When administered, they stimulate an immune reaction and confer immunity in the recipient.[40] Most vaccines operate by limiting infections at the very early stage, not necessarily by preventing them: the host immune system mediates the control and the ultimate clearance of the pathogen.[41] Many of the most widely used vaccines developed for use in humans are based on live replicating organisms that have been attenuated [41] and pathogenic organisms such as bacterial cells and viral particles that have been inactivated.[41] Vaccination with attenuated agents results in a limited infection, but recovery is usually rapid and complete.[41] The resultant immune responses are protective against fully virulent pathogens and long term immunological memory is induced.[41] The attenuated agents have a high level of immunogenicity, but they may have safety problems in immuno-suppressed patients since they contain live infectious agents.[41] Vaccination with inactivated an agent is considered safer because the vaccine contains no infectious agent.[41] However, utility is limited due to the overall low levels of immunogenicity and failure to induce the types of immune response that occur following natural infections or vaccination with attenuated vaccines. Subunit vaccines are another group of vaccines which represent a further reduction or simplification of these inactivated products since only a limited number of components from the appropriate pathogen are formulated into these types of vaccine.[41] Subunit vaccines are the safest of the three because of their defined physical and chemical characteristics.[41]

Several limitations are associated with inactivated and subunit virus vaccines and unsuccessful attempts have been made to develop products of this type against a number of viral pathogens.[41] Lack of appropriate immunogenicity is the most common limitation.[41] Hepatitis B virus vaccines also have immunogenicity limitations, leading to a failure to respond in up to 10% of the vaccinated population.[42,43] Safety problems have also been associated with some inactivated vaccines. Respiratory syncytial virus (RSV) and killed measles virus vaccines posed safety problems during their development, because they induced immune responses that enhanced pathogenesis of subsequent natural infection.[44,45]

Selection of the immunogen and delivery of this immunogen in an appropriate form are essential for the development of an effective subunit vaccine.[40] The selection of a correct immunogen will vary considerably for different vaccines, but a simple set of guidelines can be employed.[41] First, the immunogen must be an appropriate target for the immune system and induce immune responses that are effective against the pathogen.[41] Second, the choice of structure or conformation of the immunogen must be specific for the type of immune response desired: antibodies, which recognize native or discontinuous epitopes, or T lymphocytes, which recognize only small linear epitopes.[41] The conformation of the immunogen may dictate the appropriateness of the responses. This has been demonstrated for experimental HIV-1 vaccines where only native proteins induced high-titered neutralizing antibodies.[46,47] Finally, the immunogen should be of the relevant antigenic type (also called serotype) with relevance being dictated by the prevalence of different serotypes in the population.[41] A good example is the design of

influenza virus vaccines, which require modifications every year to match variation of the virus in the viral pool.[48]

It has been well established in animal studies that cytotoxic T cells (CTL) are capable of providing protection against infectious diseases and cancer, as well as controlling or eliminating these diseases once they have become established.[49] The use of antigenic peptides to elicit a CTL response has several positive features:

(i) Synthetic peptides can be readily manufactured, and they are well defined, cost effective molecules.[49]

(ii) Peptides are relatively safe molecules. Administration of an eight – to ten amino acid fragment derived from a pathogen or tumor cell offers lower safety risks than use of nucleic acids, live vectors, or recombinant proteins, which are more likely to retain inherent biological activities.[49] Their capacity to specifically manipulate the immune system, (only a few potential CTL epitopes are within a protein) and the capacity to deliver selected antigenic peptides at relatively high concentration, are important in initiating and boosting an immune response where disease already exists.[49]

Protective immunity generated by vaccination depends upon the ability of the vaccine to elicit the appropriate immune response to eradicate the antigen-bearing cell or elicit protective immunity against the antigen.[50] The use of peptide vaccines will allow the expansion of T-cell populations specific for defined immunogenic epitopes, thus allowing a more specific and potentially a more effective immune response.[50] Peptides by themselves are weak immunogens. Peptides are also rapidly degraded by peptidases and cleared by body fluids. Chemical coupling of these peptides to large proteins like ovalbumin, bovine serum albumin, keyhole limpet hemocyanin (KLH) and human gamma-

globulin called (carrier molecules) yields immunogenic peptide-carrier conjugates.[51] The advantage of the carrier proteins is that they produce chemically defined determinants, however conjugation of peptides to the protein carriers often results in modified antigenic determinants[52] and undesirable immune responses to the carrier protein have been described.[53]

In addition to the carrier proteins, adjuvants that are effective, potent and non-toxic will allow the augmentation of antigen specific immune responses.[50] Adjuvants are agents that augment specific immune responses to antigens. [54] Adjuvants may include any substance or procedure that results in a specific increase in the immunogenicity of a vaccine component.[55] Adjuvants can have three possible roles in vaccine preparation: antigen presentation, antigen targeting and immune modulation.[55]

Antigen presentation, the way in which individual antigen molecules are presented to the immune system, is of great importance for some vaccines, but of little or no relevance to others. Antigen presentation can have a major influence on the nature and magnitude of the immune response. [55] Saponin and immune stimulating complex (iscoms) have been used successfully for the development of viral vaccines, where induction of neutralizing antibodies to surface viral glycoproteins are essential. This suggests that these adjuvants are able to bind and present these antigens without modification of conformational B epitopes. [55] Retention of native conformation may be essential if protective epitopes are conformational rather than linear. For example, presentation as a cluster of molecules with hydrophilic regions outermost is beneficial for many viral antigens. [56]

Antigen targeting describes the efficiency with which the antigen is delivered to the appropriate effector cells of the immune system.[55] Targeting can incorporate a number of mechanisms, including attraction of macrophages to a tissue depot, micro–and nano-encapsulation to stimulate uptake by phagocytic cells and chemical attachment of antigen to molecules or particles bearing cell specific receptors.[55]

Immune modulation, the most complex and least understood mode of adjuvant action, describes any mechanism which modifies the processing of antigens or epitopes by immune effector cells such that either the magnitude or the nature of specific response is modified.[55]

Adjuvant stability is crucial in vaccine design. Adjuvants may degrade through chemical and physical pathways similar to vaccine immunogens.[41] Chemical degradation can occur by several mechanisms based on the chemical and physical properties of the adjuvant.[41]

A major problem for many adjuvant containing formulations is hydrolysis.[41] Small molecule adjuvants, such as muramyl dipeptide compounds, are susceptible to amide hydrolysis.[57] Saponin adjuvants, such as QS-21, have ester bonds that hydrolyze rapidly at room temperature under mildly basic (pH >7) or acidic (pH < 4) conditions.[58] Stearyl tyrosine may also be susceptible to ester hydrolysis if stored in aqueous solution at high temperature and high or low pH.[59]

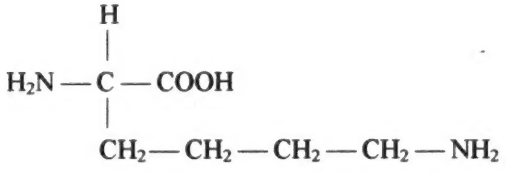
Degradation through oxidation can also be a problem,[41] especially in emulsion based formulations that are formulated using unsaturated squalene, since squalene can be highly susceptible to oxidation.[60] Physical degradation is also an issue for emulsions and liposome formulations where particle or droplet size is critical to immunogenicity[61] and

physical stability.[62] Alum, an adjuvant used extensively, is prone to physical denaturation resulting in a change of particle size or protein binding capacity which occurs when alum ages or is frozen.[63,64,65] Since the adjuvant effect of alum arises from the depoting and sustained release of antigen over time,[66] any degradation that alters the protein binding capacity can decrease adjuvant activity. The reverse situation, where protein cannot undergo normal desorption *in vivo*, can also be a problem in older alum formulations.[67,68] The consequence of hydrolysis, oxidation and physical destruction of adjuvants is a reduction in vaccine shelf life.

1.4 Design of Peptide Based Vaccines

Various avenues have been explored in the design of peptide based vaccines. Derivatization of peptides to produce prodrugs,[36] conjugation of peptides to carrier proteins,[37] and formulation of multiple antigen peptides (MAP)[37] are some of the different approaches that have been tried. The MAP format for peptide design is of particular interest for this project and will now be discussed in detail.

Multiple antigen peptides have chemically defined structures that can be prepared directly through the solid-phase synthesis of a peptide antigen onto an oligomeric lysine core.[69] Lysine is used because it has two functional (amino) groups available for branching reactions, the alpha (α) and epsilon (ε) amino groups. The structure of lysine is shown below:



The key feature of the MAP system is the amplification of peptide antigen in a chemically defined manner.[70] The MAP system is prepared by utilizing a core matrix that consists of several sequential levels of tri-functional amino acids as a scaffold.[71] The first level of coupling produces two reactive amino ends to give a bivalent MAP (Fig.5). Further sequential propagation of lysine produces MAPs containing tetravalent or octavalent reactive amino ends, which are highly immunogenic.[70]

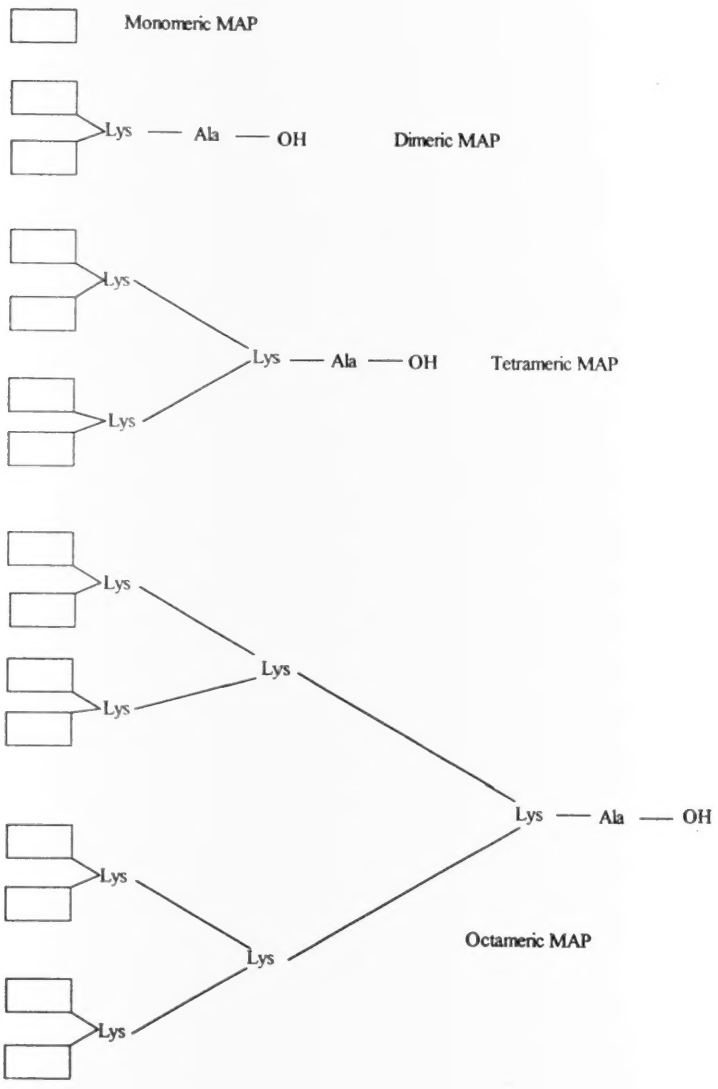


Figure 5. Schematic representation of MAP constructs, showing the monomeric, dimeric, tetrameric and octameric MAP constructs.

In the production of a MAP, the peptide antigen, as well as the core matrix, are synthesized in a continuous sequence by stepwise solid-phase chemistry using standard Boc-benzyl chemistry or Fmoc-tert-butyl chemistry. [72,70] A conventional method of coupling mediated by dicyclohexylcarbodiimide / 1 - hydroxy benzotriazole in dimethyl formamide is then used to produce the MAP structure. [70] MAP resins are treated with deprotecting reagents, then the peptides are cleaved from the resins with low/high hydrogen fluoride (Boc chemistry) or with 95% trifluoroacetic acid (Fmoc chemistry). The crude MAPs are then extracted with urea and dialyzed with suitable buffer. The resulting molecule contains multiple antigenic determinants surrounding the small branching lysine core in high-density clusters. [70]

MAP peptides have the potential to be used as synthetic vaccines, as antigens for solid phase immunoassays and to raise antibodies for immunocytochemistry. [73] MAP constructs have also been used to generate immune sera that specifically recognize the native proteins and are characterized by titers considerably higher than the sera prepared from the same peptide anchored covalently to key hole limpet haemocyanin as a carrier. [71] MAPs have further been used in the preparation of experimental vaccines against hepatitis, [74] malaria, [75] and foot-and-mouth disease. [76]

It has been reported that peptides in the MAP format may have *in vivo* half-lives that are longer than those of unconjugated linear peptides, [70] and the difference in conformation in solution between linear and MAP peptides likely contributes to their observed differences in immunogenicity. [72] Some of the advantages of MAP, both physical and immunological will now be discussed briefly.

(i) Physically, there is no need for conjugation to carrier proteins, thus avoiding the potential consequences of epitopic suppression related to the carrier protein.[70] MAPs are safe, non-replicating chemically defined molecules.[70] MAP peptides exhibit the flexibility of incorporating multiple epitopes.[70] They are cheap, lyophilized chemical products and are stable without refrigeration.[70]

(ii) Immunological advantages of MAP include the immunization against predetermined antigenic protein sequences and the avoidance of the stimulation of immune responses against suppressor or non-protective immunodominant sequences present in the original protein.[70] There is also the generation of a long-lasting antibody response and enhancement of specific B- and T-cell responses through the presentation of more than one immunogenic epitope.[70] MAP constructs also have the inherent feasibility of overcoming genetic unresponsiveness by means of the incorporation of universal T - helper cell determinants,[70] and the possibility of overcoming MHC - associated non-responsiveness.[70]

B. Hypothesis

We postulate that peptides in the MAP (MULTIPLE ANTIGEN PEPTIDE) format have *in vivo* half lives that are longer than those of unconjugated linear peptides, hence increasing their biological effectiveness when formulated as vaccines.

Biotin-linked peptide will tend to be more stable than the free peptide in biological fluids due to the steric protection at the N - terminus afforded by the biotin moiety which prevents the peptide from being degraded by N- terminal peptidases.

Formulation of peptides into PLGA microspheres will increase their stability in biological fluids due to their entrapment in the inner core of the microsphere. It is postulated that this leads to their protection by the outer biodegradable polymer shell that surrounds the core, reducing the chances of destruction of the peptides by the peptidases in the body.

C. Experimental

3.1 Materials

3.1.1 Peptides, Chemicals and Solvents

Peptides were obtained from Biomira Inc., (Edmonton, Alberta) and used without further purification. These peptides (> 90% purity) include human MUC1 peptide (BP1-023) [PDTRPAPGSTAPPAHGVTSA], molecular weight: 1887.01 g/mole; Human MUC1 MAP-4 (BP1-026) [(PDTRPAPGSTAPPAHGVTSA) 4 MAP], molecular weight: 7882 g/mole; Ova $_{257 - 264}$ (BP1-053) [SIINFEKL], molecular weight: 963.15 g/mole; Ova $_{323 - 339}$ (BP1-037) [ISQAVHAAHAEINEAGR], molecular weight: 1774.9 g/mole and Biotin linked Ova $_{323 - 339}$ (BP1-038) [BIOTIN-ISQAVHAAHAEINEAGR], molecular weight: 2114.39 g/mole. Chloroform, Fisher Scientific (Nepean, Ontario); acetonitrile and water, BDH Caledon, (George town, Ontario) and trifluoroacetic acid (TFA), Sigma, (St. Louis, Missouri, U.S.A.) were all HPLC grade and used without further purification. Trichloroacetic acid (TCA) was purchased from BDH Caledon, (George-town, Ontario). The composition of one liter of PBS is NaCl, 154 mM, NaH_2PO_4, 1.9 mM, and $NaHPO_4$, 8.1 mM. All reagents were purchased from BDH Caledon, (George-town, Ontario) and used without further purification. Polyvinyl alcohol (PVA), 87-89% hydrolyzed, average molecular weight: 31,000-50,000 g/mole and poly (d,l-lactic acid-co-glycolic acid)[PLGA] 50/50, molecular weight: 60,000 g/mole, were purchased commercially from Aldrich , (Milwaukee, Wisconsin, U.S.A.) and BPI, (Birmingham, Alabama, U.S.A.) respectively and used without further purification. Human serum, normal mouse serum (MS) from healthy mice were bought from Chemicon International Inc. (Temecula,

California U.S.A.). The MS was aseptically frozen in aliquots at -20°C and used when necessary.

3.2 Methods

3.2.1 Serum Preparation from Human Blood

Blood was collected at the University of Alberta Hospital, in the outpatient laboratory by venipuncture in a Corvac integrated serum separator tube (HRI 8881-302015), and stored at 4°C overnight to allow the clot to retract. The serum was then transferred to a clean tube and centrifuged at 4000 rpm for 10 minutes in a Mistral 2000 centrifuge (Loughborough, Leicestershire, England) to pellet the blood cells. The supernatant was aseptically frozen at -20°C in aliquots of 2 ml until required.

3.2.2 HPLC Analysis of Peptides

Reverse-phase high-pressure liquid chromatography (HPLC) using a Waters 625 LC system, (Milford, Massachusetts, U.S.A), was used to analyze all the peptides in this study. The samples were analyzed at room temperature with a Waters 15μm, 8×100mm Nova-Pak C_{18} column. The mobile phases used were 0.1% TFA in 100% water (solution B) and 0.85% TFA in 70% acetonitrile (solution C). A 10-48% gradient of solution C, over a period of 22 minutes was used to elute human MUC1 peptide in both human serum (HS) and mouse serum (MS), and human MUC1 MAP-4 peptide in HS. A 17-83% gradient of solution C over a period of 22 minutes was used for human MUC1 MAP-4 in MS. A 9-51% gradient of solution C over a period of 22 minutes was used for Ova $_{323-339}$

in HS. A 20-48% gradient of solution C over a period of 40 minutes was used for biotin-linked Ova $_{323-339}$ in HS. A 19-61% gradient of solution C over a period of 25 minutes was used for Ova $_{257-264}$ in HS. The flow rate was set at 1ml/min and with detection using a Waters 486 tunable absorbance detector set at 210 nm. The AUFS (absorbance unit full scale) was set at 0.05 and a Waters 746 data module integrator was used to quantify peak intensities. A standard curve was generated for each of the peptides studied by choosing concentrations ranging from 0.0025 mg/ml to 0.05 mg/ml (0.5µg/200 µl to 10µg /200 µl), and using two injections for each concentration. Kinetic analyses were carried out by least-square analysis of the time variation of the log of the integration of peak area against time. When necessary, corrections were made for small interfering peaks contained in the serum.

3.2.3 Validation of Peptide Analysis in Serum

A 0.025 mg / ml peptide standard was run to calibrate the column and to determine the concentration of the peptide for the day. Two types of recovery experiments were carried out. First, human (HS) or mouse (MS) serum (1 ml respectively) was equilibrated for 15 minutes at 4°C, then 500 µl serum respectively was precipitated with 6% TCA (1 ml). The resulting solution was allowed to cool at 4°C for 15 minutes. The solution was then centrifuged in Eppendorf tubes at 14,000 rpm for three minutes. The resulting supernatant was collected and exact volumes spiked with exact amounts of peptide. Each sample was then resolved by HPLC. This was done to determine if the peptides could be detected by HPLC in the absence of the serum proteins and to check the reproducibility of the assay. Furthermore, each experiment was done in duplicate. The second recovery

experiment was done to ascertain if peptides could be detected by HPLC before precipitation of the serum proteins. This was used to show that the peptides are not extracted with other serum proteins. HS or MS (2 ml) was equilibrated at 4°C for 15 minutes, then the peptide (10 µl) was added to the equilibrated serum and vortexed. The reaction was stopped by adding the serum-peptide (200 µl) mixture to 6% TCA (400 µl) and vortexed again. The solution was allowed to cool for 15 minutes at 4°C and then centrifuged in Eppendorf tubes at 14,000 rpm for three minutes. The volume of the supernatant was measured and 250 µl of the sample was injected into the HPLC. This recovery experiment was done in duplicate.

3.2.4 Peptide Degradation Kinetics

The reaction media consisting of 2 ml of either HS or MS were equilibrated at 37°C for 15 minutes, after which 10 µl peptide stock solution (10 mg/ml) in DMSO was added to give a final concentration in serum of 50 µg/ml. At known time intervals, 200 µl of the reaction mixture (peptide plus either HS or MS) was removed and added to 400 µl of 6% aqueous TCA. The solution was cooled at 4°C for 15 minutes and then centrifuged at 14,000 rpm for three minutes. This resulted in a precipitation of serum proteins.

3.2.5 Formulation of Microspheres

Chloroform (500 µl) was added to poly (d,1-lactic acid–co-glycolic acid (PLGA) (100 mg), and shaken for 10 minutes to dissolve the PLGA. 8 ml of polyvinyl alcohol (PVA) (9% w/v) in double distilled ionized water (ddH_2O) was measured into a beaker and stirred with a magnetic stirrer in a fume hood. 150 µl of $Ova_{257-264}$ peptide (1 mg/ml) was added to the vial containing the PLGA, shaken vigorously for 10 sec. and then sonicated for another 10 seconds (level four) using the ultrasonic processor XL heat systems, (Farmingdale, New York, U.S.A.). A thick milky white emulsion was formed, considered to be the primary emulsion. 2 ml of PVA (9% w/v) was added to the primary emulsion and shaken vigorously for 10 seconds and sonicated for 20 seconds (level two). A thick emulsion formed again. This is called the secondary emulsion. The secondary emulsion was transferred drop-wise using a Pasteur pipette into the PVA (8 ml 9% w/v) and stirred for two hours to allow evaporation of the chloroform. This mixture was centrifuged at 20,000 rpm at 20°C for 10 minutes using the Beckman model L8-55 ultracentrifuge, (Palo Alto, California, U.S.A). The supernatant was decanted and the pellet re-suspended with double distilled ionized water (ddH_2O). The pellet was washed two times and centrifuged at 20,000 rpm for five minutes each time. The pellet was re-suspended in a minimal volume of ddH_2O, then transferred into a round bottom flask. The microspheres were quickly frozen in an acetone /dry ice bath and the contents freeze-dried in the Labconco freeze dryer (Kansas, Missouri, U.S.A.) for two to three days.

To determine the encapsulation efficiency, the microspheres were analyzed for peptide content using HPLC. Microspheres (10-50 mg) were dissolved in chloroform (1 ml), then ddH_2O (1 ml) was added and stirred for 20 minutes. The mixture was then

transferred to a polystyrene tube and centrifuged for 10 minutes at 1000/g in the Mistral 2000 centrifuge (Loughborough, Leicestershire, England). Of the three layers formed after centrifugation, the top and middle layers were collected into an Eppendorf tube and further centrifuged at 14,000 rpm for 10 minutes in an Eppendorf microcentifuge 5415C (Rexdale, Ontario). The supernatant was then injected into the reverse phase HPLC column, using a Waters 625 LC system to check the presence of any peptide in the fraction. The chloroform layer was evaporated to dryness with N_2 gas then dissolved in ddH_2O (1 ml) and shaken for 30 minutes, and then injected into the HPLC column.

3.2.6 Release of Ova $_{257-264}$ Peptde Encapsulated in PLGA Microspheres in 100% HS

PLGA microspheres containing approximately 30 µg peptide were used in these experiments. This weight was based on the encapsulation efficiency as described in section 3.2.2. Microsphere containing the peptide were placed in 1 ml HS. As a control, the same quantity of microspheres was placed in 1ml phosphate buffered saline (PBS). Both mixtures were vortexed and incubated with continuous shaking at 37°C. At various time intervals (5,15, 30, 60 and 300 min.) the samples were removed and centrifuged at 14,000 rpm for 10 minutes in Eppendorf tubes. Supernatants from each tube were removed and 200 µl of the HS was precipitated with 400 µl of 6% TCA. The PBS and TCA supernatants were later analyzed respectively. The pellets from above were extracted with chloroform-water (1:1 volume to volume) as previously described in section 3.2.5.

D. Results and Discussion

4.1 Validation of Assay

The validation of the peptide degradation kinetic assay was carried out to ascertain if the approach developed for this study was suitable and reproducible. Reverse phase HPLC is the most widely used mode of HPLC used in peptide separations because of its ability to separate peptides of closely related structure.[4] The flexibility of reverse phase chromatography parameters such as pH, mobile phase, and temperature which can be varied for optimum resolution also adds to it's advantage as being a good technique for the separation of one peptide from a complex mixture. [4] The area under the curve is also useful for the quantification of these separated peptides. Reverse phase HPLC has been used to study peptide stability in serum and other biological media by various researchers.[35,77,78,79] Their experiments have shown that this technique is suitable for this kind of stability study. Other advantages of reverse phase HPLC include reproducibility of the hydrophobic bonded phase columns; availability of the mobile phase, water and mobile phase modifiers for example, acetonitrile and methanol the most commonly used ones; elution order is often predictable based on the degree of hydrophobicity of the solute molecule.[4] Reverse phase HPLC however has suffered from problems associated with denaturation including loss of activity, poor recoveries, broad misshapen peaks and ghosting (a residual peptide peak from a previous run) .[80] Although some of these problems can be directly related to the column being used,[81] they can be overcome by selective optimization of sample pretreatment and mobile phase and hardware considerations.[82]

In the experiments presented here, the area under the curve of the peptide peak from the UV (210 nm) elution trace, was used for the quantification of peptides. The calibration curves for the human MUC1 and human MUC1 MAP-4 peptides were generated with concentrations ranging from. 0.005 mg/ml to 0.05 mg/ml (1 μg/200 μl to 10 μg /200 μl), with two injections for each concentration. The r^2 values obtained were 0.999 and 0.999 respectively. For Ova $_{257-264}$, Ova $_{323-339}$ and biotin linked Ova $_{323-339}$ peptides, the calibration curves were generated with concentrations ranging from 0.0025mg/ml to 0.05mg/ml (0.5 μg/200 μl to 10 μg /200 μl). The r^2 values obtained were 0.999, 0.998 and 0.999 respectively. Error bars are present but may be too small to be seen at some points. These r^2 values obtained were close to 1.00, showing that the area under the elution peaks were concentration dependent. These calibration curves were used for quantification of the peptides in the serum samples. The calibration curves are shown in figures 6a and 6b.

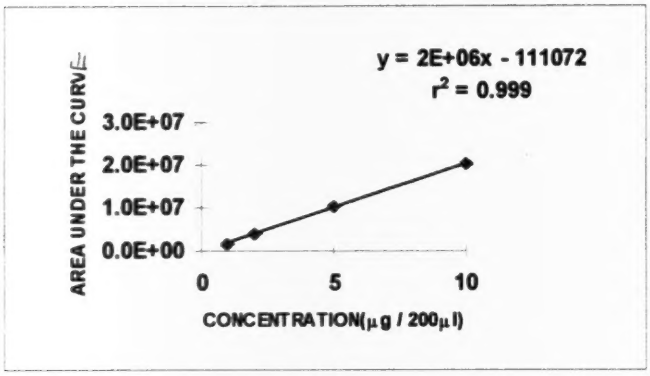
Serum proteins would be expected to interfere with the HPLC assay. Therefore removal of most of the protein from the serum is required before HPLC analysis. Reagents such as ethanol, methanol and trichloroacetic acid have been used to precipitate serum proteins in sample preparation for HPLC. The above reagents were examined for suitability for sample preparation for HPLC by first precipitating the serum samples with them and by using the supernatant as the sample matrix. The supernatants were spiked with a known quantity of peptide and the peptide peak and the matrix effect was examined for quantitative recovery of the peptides. Among the three reagents, 6% trichloroacetic acid gave the least interference and the best peptide recovery.

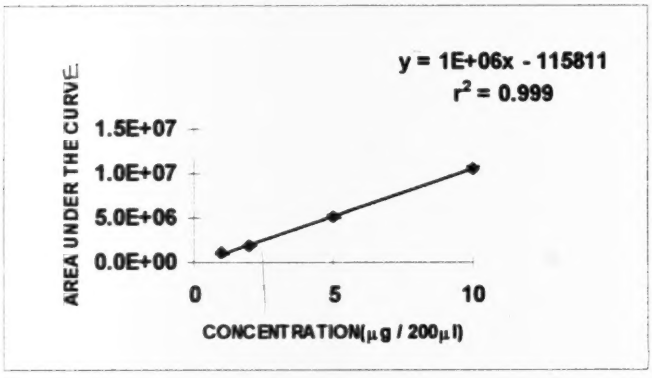
Table 2 shows the percentage of peptide recovered after precipitation of the human serum to ensure that the peptide could be fully recovered in the absence of serum proteases. The percentage recovery obtained for human MUC1, human MUC1 MAP-4 and Ova $_{323-339}$ peptides were 95.4±1.3%; 97.0±3.5%; 90.0±3.8% respectively. These results indicate that the removal of serum proteins with 6% TCA was acceptable for sample preparation for peptide HPLC. The peptide assay was further validated by examining the recovery of peptide from serum at 'zero' time. For this purpose serum samples were spiked with the peptides and serum proteins were precipitated immediately with 6% TCA. The results of the peptide recovery based on HPLC assay are shown in Table 3. A recovery of 97.1±2.0% was obtained for the human MUC1 peptide and a recovery of 94.3±4.1% was obtained for the human MUC1 MAP-4 peptide. Ova $_{323-339}$ peptide had a recovery of 93.3±1.8%. These percentage recoveries indicated the suitability of the conditions for the study. Tables 4 and 5 show the recoveries for the human MUC1 and human MUC1 MAP-4 peptides after and before precipitation of mouse serum. The results obtained indicate that the conditions for the assay are suitable for the study.

From the results shown in tables 2- 5, it can be seen that the percentage recovered was generally higher with peptides spiked in serum free of proteases than that of peptides spiked in serum with proteases. This may indicate that there are a lot of proteases in the serum which degrade the peptides as soon as they come in contact with them, since the reaction was stopped almost immediately after the peptides were added to the serum. However, it will be seen in the peptide degradation kinetic study, that the degradation of the human MUC1 peptides in serum with proteases did not proceed at a fast rate as did the Ova peptides. This can be attributed to the position of the imino acid residue proline in

the peptide sequence which decreased the rate at which the proteases degraded the peptide, hence having a longer half-life. The Ova peptides had a shorter half-life, but the peptide was degraded gradually.

For the Ova $_{257-264}$ peptide, at time zero, the recovery obtained was below 80%, indicating that the peptide was either adsorbing to the walls of the Eppendorf tube due to its hydrophobic nature or the peptide was bound to some serum protein and extracted during the precipitation using 6% TCA. However, the recovery obtained after spiking the peptide in serum free of proteases was 98.6±3.0%. This suggests the Ova peptide may be quickly broken down by proteases in serum since the recovery obtained after the spike was over 80%. This low recovery was also observed for the biotin linked Ova $_{323-339}$ peptide, but in this case, the addition of the biotin group may have been responsible for the low recovery. As will be shown in the peptide degradation kinetic study, the Ova $_{323-339}$ peptide appears to be more stable when linked to biotin at the N-terminus than when it is not. (The percentage recovered in the peptide degradation kinetic study was corrected to the amount recovered after the spike at time zero). In contrast, M.F. Powell and colleagues, in 1992, reported a recovery of ≥ 85% for all peptides studied in HS (for both spike and recovery at zero min),[77] using similar methodology.





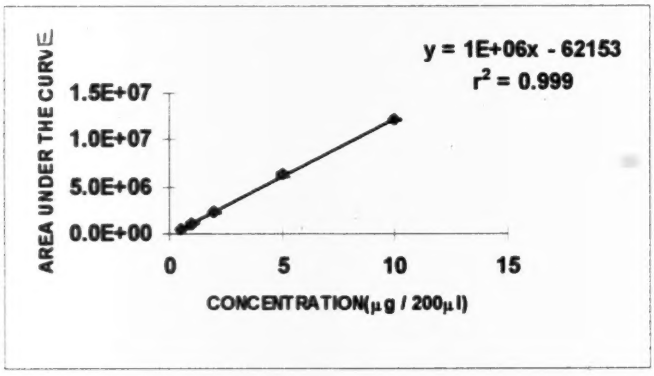
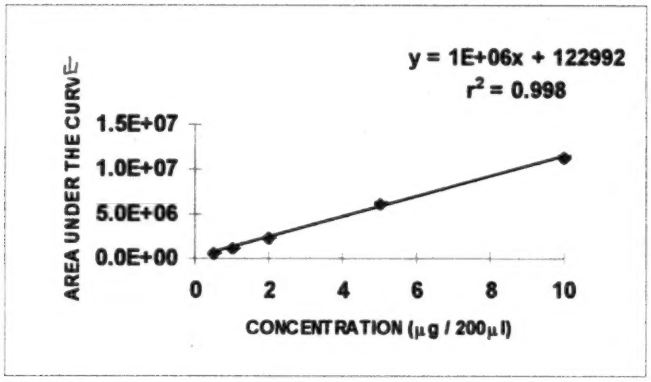


Figure 6a. Calibration curve for peptides A (human MUC1), B (human MUC1 4-MAP), C (Ova $_{257-264}$).



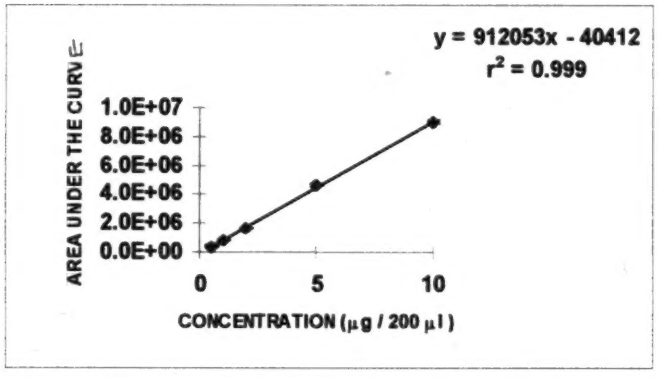


Figure 6b. Calibration curve for peptides D (Ova $_{323-339}$) and E (Biotin-Ova $_{323-339}$).

The results obtained for recovery after precipitation of HS proteins and recovery at time zero are shown in Tables 2 and 3. Table 4 and 5 show the recovery after precipitation of MS proteins and recovery at time zero.

Table 2. Recovery of peptides A-E after precipitation of 100% HS to ensure that the peptide could be fully recovered in the absence of serum proteases.

Peptide	Percent recovery
A. Human MUC1 peptide	95.4±1.2%
B. Human MUC1 MAP-4 peptide	97.0±3.5%
C. Ova $_{257-264}$ peptide	98.6±3.0%
D. Ova $_{323-339}$ peptide	90.0±3.8%
E. Biotin-Ova $_{323-339}$ peptide	96.6±1.9%

Table 3. Recovery of peptides A-E at time zero in 100% HS to ascertain that the peptide could be detected after the action of serum proteases.

Peptide	Percent recovery
A. Human MUC1 peptide	97.1±2.0%
B. Human MUC1 MAP-4 peptide	94.3±4.1%
C. Ova $_{257-264}$ peptide	71.3±3.1%
D. Ova $_{323-339}$ peptide	93.31±1.8%
E. Biotin-Ova $_{323-339}$ peptide	60.8±1.3%

Table 4. Recovery of peptides A and B after precipitation of 100% MS to ensure that the peptide could be fully recovered in the absence of serum proteases.

Peptide	Percent recovery
A. Human MUC1 peptide	88.8±2.3%
B. Human MUC1 MAP-4 peptide	95.8±1.7%

Table 5. Recovery of peptides A and B at time zero in 100% MS to ascertain that the peptide could be detected after the action of serum proteases.

Peptide	Percent recovery
A. Human MUC1 peptide	102.4±1.3%
B. Human MUC1 MAP-4 peptide	100.0±4.3%

4.2 Kinetics of Peptide Degradation

4.2.1 Effect of MAP Clustering on Peptide Stability in Serum

Degradation kinetic studies of human MUC1 and human MUC1 MAP-4 peptides were carried out in both HS and MS respectively at 37°C as described in section 3.2.4. This was done to determine the apparent half-lives of the peptides in both types of serum. The results obtained from this are indicated in figures 7-10.

The apparent half-life obtained for the human MUC1 peptide in human serum was 38.5 hours (fig. 7) compared to human MUC1 MAP-4 peptide, ($P=1.503 * 10^{-6}$) which had a half-life of 128.3 hours (fig. 9). These results indicate that formulation of peptide in the MAP format increases the overall stability of the peptide as was suggested in our hypothesis (section B).

It has previously been reported by some researchers that peptide sequences with proline either at the N-terminal or penultimate position of the N- terminal usually have a prolonged half life.[83] This is due to the unique structure of the imino acid proline (section 1.2.2) which makes the peptide bonds, (Xaa-Pro or Pro-Xaa, where X is an amino acid) more resistant to metabolism.[84,85] The half-life of MUC1 peptide (PDTRPAPGSTAPPAHGVTSA) was longer than expected, and the presence of proline at the N-terminal may account for this. Also the complete absence, or trace, amounts of the proline specific peptidases in HS may account for the increased half-life. Proline may be an essential amino acid residue in the human physiological process, so that the production of large quantities of proline specific peptidases is controlled. Proline has been suggested to serve as an important regulatory signal in determining the life time and degradation rates of biological active peptides.[86] It may be suggested that the imino acid

residue proline can have some positive effects in vaccine development. Inclusion of proline at the N-terminal or the penultimate position will stabilize a peptide formulated as a vaccine.

Peptides containing proline at the terminal or penultimate position at the N-terminal region have been reported to degrade faster in rat serum.[78,79] Metabolism of the endogenous brain peptides Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH_2) and MIF-1 (Pro-Leu-Gly-NH_2) has been evaluated by HPLC analysis. Incubation of the titrated peptides in human and rat plasma, indicated that in the rat plasma, more than half of the intact Tyr-MIF-1 and MIF-1 was degraded within five minutes.[86] In contrast to this, it took five days for 50% of MIF-1 to be degraded in human plasma.[86] Tyr-MIF-1, was also reported to be rapidly degraded in the plasma from both species.[78] Similar results have been shown for H-Pro-[^{3}H]-Leu-Gly-NH_2 (PLG).[79] After 1 hr and incubation at 37°C, there was no observable change in proteolysis and the half-life was determined to be 5.6 days.[79] However *in vitro* degradation of PLG was reportedly more rapid in rat plasma.[79] A complete degradation was reported after 1 hour at in rat serum while 80% degradation was observed in rat plasma.[79]

A preliminary study of the stability of the human MUC1 and the human MUC1 MAP-4 carried out in mouse serum and rat serum showed that both sera behaved similarly. In light of the above preliminary study and the above reports, the stabilities of the human MUC1 and human MUC1 MAP-4 were evaluated in mouse serum that is more readily available. The results obtained (figures 7 and 9), show that these peptides were degraded faster in MS than in HS. The apparent half-life obtained for the human MUC1 peptide in MS was 17.7 hours, while that obtained for the MAP peptide was 55.0 hours

(P=0.0031). Our study thus showed that the formulation of MAP peptides increases the stability of the peptides. This is the first time such a study has been reported.

Although increased immunogenicity of MAP constructs compared to linear peptides has not been fully characterized, linear peptides which were ineffective in raising antibodies have been reported to become antigenic when given in MAP formats.[87] In addition, antibody titers induced by MAP peptides have also been shown to be superior to those obtained by the corresponding linear form of the peptide conjugated to keyhole limpet hemocyanin (KLH).[71] B. Nardelli and colleagues have also demonstrated in a study the effectiveness of MAP in eliciting antibody response against sequences of gp 120 V3 region, without the need for conjugation to a protein carrier.[39] The ability of MAP to attain a polymeric and macromolecular structure when compared to a linear peptide, which may also be cleared at a faster rate from the body, has been reported to be a possible explanation for increased immunogenicity.[70] From the results shown in our study, the greater stability of the MAP –4 peptide in serum may be a major reason for increased immunogenicity.

A mass spectrum of the peptides before and after incubation in HS and MS was run using a positive mode electrospray ionization technique with a cone voltage of 45V (Fisons VG Trio 2000, Altrincham, United Kingdom). This technique has the advantage of allowing rapid, accurate and sensitive analysis of a wide range of analytes. The peptides produce a series of multiply charged ions and the acquired data can be transformed by a data system to give a molecular weight profile of the biopolymer. (This information was obtained from the Fisons VG Trio 2000 manual). This was done to ascertain that the peptide peaks detected after incubation have the same mass properties as the native

peptides. The mass spectra obtained for the human MUC1 peptide showed that the peptide detected after incubation in serum was identical to the native peptide. Table 6 below shows the m/z values obtained.

Table 6. m/z values obtained for the human MUC1 peptide.

	Native peptide	Before incubation	After incubation in human serum	After incubation in mouse serum
m	1887.01	Not seen	Not seen	Not seen
$m/_2$	943.5	943.5	944.4	945.1
$m/_3$	629.0	629.6	630.1	630.6

The mass spectra are shown in appendix B. (A mass spectrum of the human MUC1 MAP-4 peptide was also run, but it was not possible to obtain a mass both for the native peptide and peptide after incubation in serum. This might be due to the difficulty or inability of the MAP peptide to be ionized. The result is shown in appendix C).

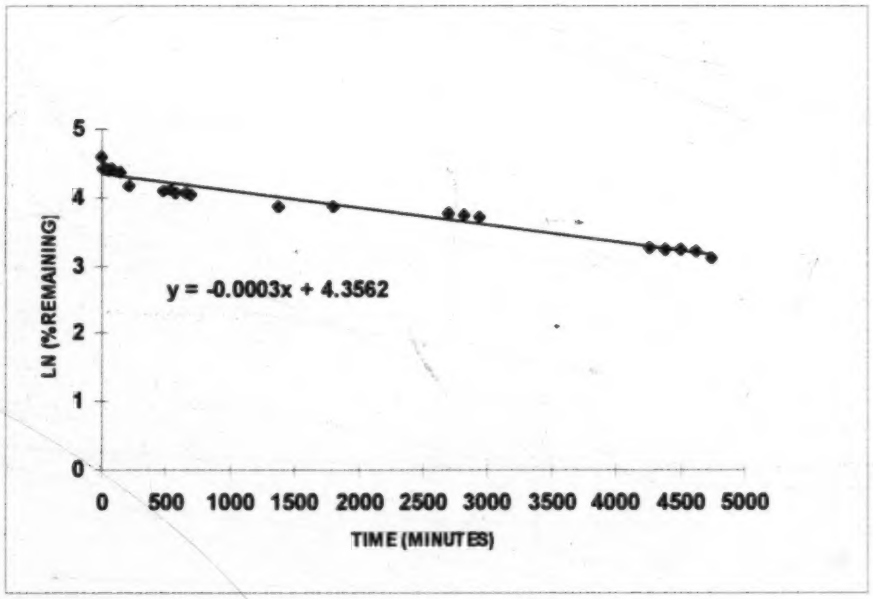


Figure 7. Kinetics of degradation of the Human MUC1 peptide in 100% HS.

This degradation curve was obtained from HPLC analysis (section 3.2.2). The values are means of percentage recovered, (n=4). The equation for the straight line was $y = -0.0003x + 4.3562$. The apparent half-life was calculated using the equation: $T_{1/2} = 0.693 / K$, where K is the slope of the line. The apparent half-life was calculated to be 38.5 hours. The standard error for the percentage recovered ranged from 0.1 to 2.7.

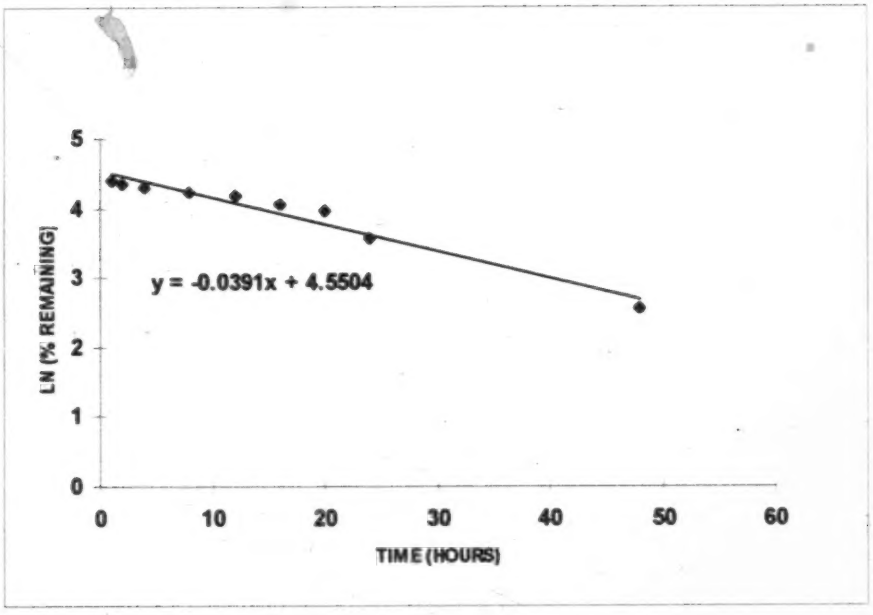


Figure 8. Kinetics of degradation of the Human MUC1 peptide in 100% MS.

This degradation kinetic was obtained from HPLC analysis (section 3.2.2). The values are means of percentage recovered, (n=4). The equation for the straight line was $y = -0.0391x + 4.5504$. The apparent half-life was calculated using the equation: $T_{1/2} = 0.693 / K$, where K is the slope of the line. The apparent half-life was calculated to be 17.7 hours±0.4. The standard error for the percentage recovered ranged from 0.2 to 0.9.

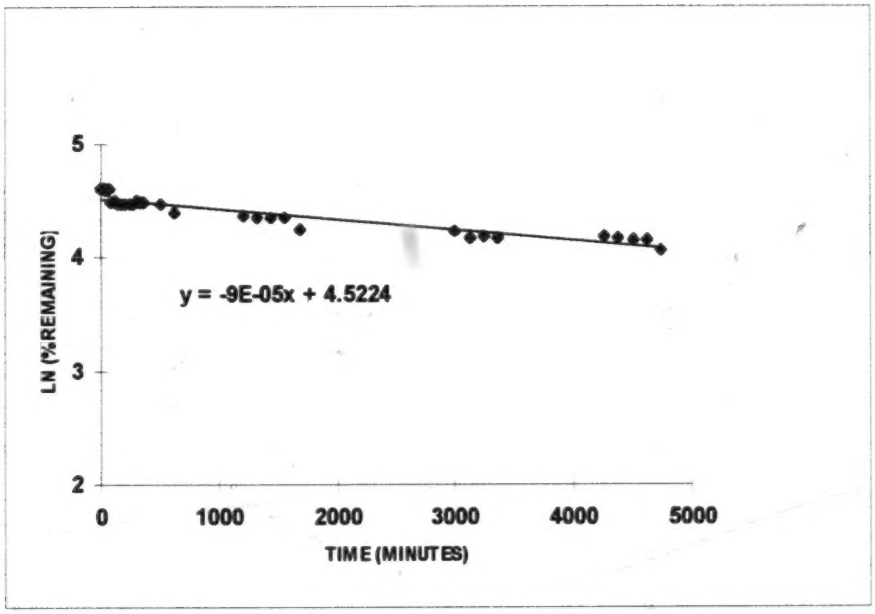


Figure 9. Kinetics of degradation of the Human MUC1 4- MAP peptide in 100% HS.

This degradation curve was obtained from HPLC analysis (section 3.2.2). The values are means of percentage recovered, (n=4). The equation for the straight line was $y = -0.00009x + 4.5225$. The apparent half-life was calculated using the equation: $T_{1/2} = 0.693 / K$, where K is the slope of the line. The apparent half-life was calculated to be 128.3 hours. The standard error obtained for the percentage recovered ranged from 0.6 to 1.5.

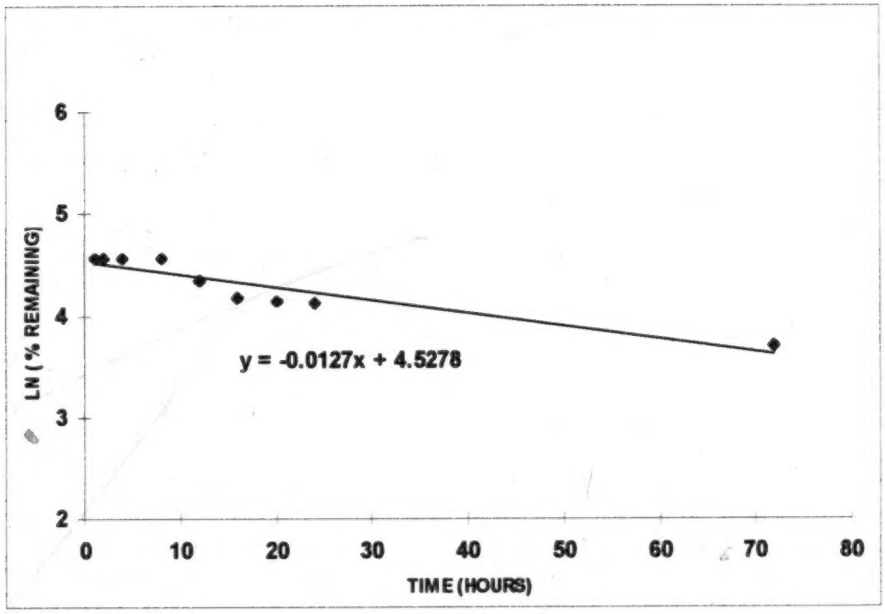


Figure 10. Kinetics of degradation of the Human MUC1 4- MAP peptide in 100% MS.

This degradation curve was obtained from HPLC analysis (section 3.2.2). The values are means of percentage recovered, (n=4). The equation for the straight line was $y = -0.0127x + 4.5278$. The apparent half-life was calculated using the equation: $T_{1/2} = 0.693 / K$, where K is the slope of the line. The apparent half-life was calculated to be 55.0 hours±1.0. The standard error obtained for the percentage recovered ranged form 0.6 to 1.9.

4.2.2 Effect of Encapsulation of Ova $_{257-264}$ Peptide in PLGA Microspheres on Peptide Stability in Serum

Synthetic peptides are poor immunogens thus formulations or adjuvants are required to increase their immunogenicity when formulated as vaccines

. The controlled delivery of tetanus toxoid in polylactic acid (PLA) or PLGA microspheres has been reported to greatly enhance its immunogenicity.[88] One possible mechanism with which these PLA or PLGA microspheres may operate is that they allow localized targeted delivery of the vaccine to antigen presenting cells and the lymphatic system.[89] They may also act by protecting the peptides from peptidases in the body fluids, based on the assumption that the peptide is inside the core of the microsphere. The dose range for peptides encapsulated in PLGA microspheres to be immunogenic has been shown in studies in our lab to be in the nanogram level, thus the amount of peptides needed is greatly reduced. The effect of PLGA microsphere formulation on the effect of peptide stability using a model ovalbumin peptide (Ova $_{257-264}$) was chosen.

Polyesters of lactic and glycolic acids respectively and their co-polymers are usually selected as appropriate materials for formulation.[89] This is because they have been approved by the U.S. Food and Drug Administration for a number of clinical applications, such as surgical sutures.[90,91] The polymer undergoes random, non enzymatic hydrolysis of its backbone ester linkages *in vivo* at a rate controlled by the polymer's molecular weight, matrix surface area, monomer steroregularity and ratio of lactic to glycolic acid.[89] The Ova $_{257-264}$ peptide contains the H-2K^b binding motif and is the ovalbumin CTL epitope for H-2^b mice. Further, the preliminary study showed that this peptide is very rapidly degraded by serum peptidases. A kinetic study involving degradation of Ova $_{257-264}$ peptide in HS was

carried out as described in section 3.2.4, to determine if the apparent half-life would be shorter than that of the human MUC1 peptides that had a considerably longer half-life due to the presence of the imino acid residue proline. The results show a 68% recovery after 45 sec, and after 645 sec. no peptide was recovered indicating a very short half-life of 2.5 minutes±0.076. The results are shown in Figure 11.

The most common buffers used in release studies involving microspheres are PBS [90] and Tris buffer (pH 7.4).[93] However, our study was carried out in HS with PBS as control. This was done to allow comparison of the data to those obtained from the stability studies involving the peptide alone in HS. The results obtained from these studies are indicated in Figures 12 and 13. No peaks were observed for the peptides in serum supernatant, since they are most probably degraded immediately after they are released into the human serum. A gradual increase in the release profile was however observed for PBS (Figure 12). From the results shown in Figure 12 (control study), it is evident that there is a problem. The sum of the amount of peptide released in the PBS supernatant and the amount remaining in the pellet is about 65% as opposed to close to 100% as would be expected. This poor recovery can be attributed to a variety of effects such as a burst effect where by the Ova $_{257-264}$ peptide is released in large quantities and being hydrophobic is stuck on the walls of the tube. Also due to lack of sufficient quantities of microspheres, this study could not be repeated and only three time points were studied in the control, and priority given to the serum study where more time points were studied. The results obtained show that the encapsulation of the Ova $_{257-264}$ into PLGA microspheres increased its stability. A half-life of 330 minutes was obtained for the encapsulated Ova $_{257-264}$ compared to a half-life of 2.5 minutes obtained for the single Ova $_{257-264}$ peptide.

The mass spectra obtained for the Ova $_{257-264}$ peptide showed that the peptide detected after incubation in serum is identical to that of the native peptide. Table 7 shows the m/z values obtained.

Table 7. m/z values obtained for the Ova $_{257-264}$ peptide.

	Native peptide	Before incubation	After incubation in human serum
m	963.5	964.7	965.5
$m/_2$	481.8	483.0	482.9

The mass spectrum is represented in appendix A.

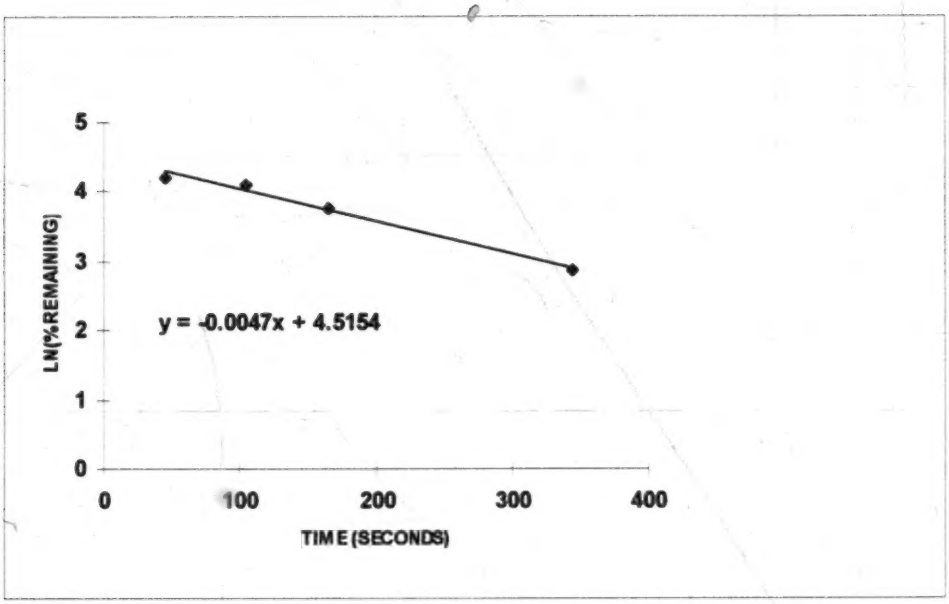


Figure 11. Kinetics of degradation of the peptide Ova $_{257-264}$ in 100% HS.

This degradation curve was obtained from HPLC analysis (section 3.2.2). The values are means of percentage recovered, (n=4). The equation for the straight line was $y = -0.0047x + 4.5154$. The apparent half-life was calculated using the equation: $T_{1/2} = 0.693 / K$, where K is the slope of the line. The apparent half-life was calculated to be 147.5 sec.±4.7. The standard error for the percentage recovered ranged from 0.2 to 1.7.

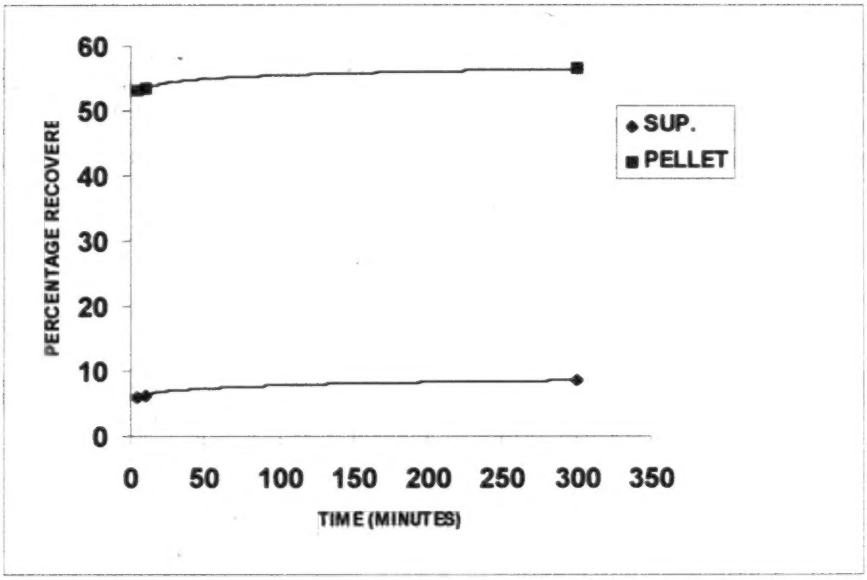


Figure 12. Release study of Ova $_{257-264}$ peptide loaded PLGA microspheres in PBS. The method for the release study is described in section 3.2.6.

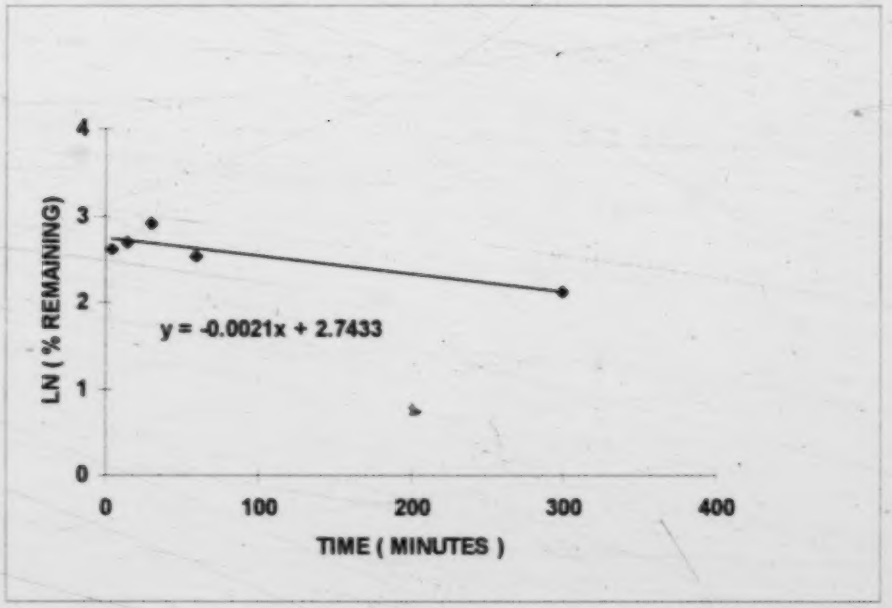


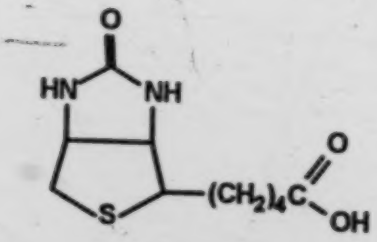
Figure 13. Kinetics of degradation of Ova $_{257-264}$ peptide loaded PLGA microspheres in 100% HS.

The equation for the straight line: $y = -0.0021x + 2.7433$. The apparent half life was calculated using the equation: $T_{1/2} = 0.693 / K$, where K is the slope of the line. The apparent half-life was calculated to be 330 minutes. Values are mean ± range, since only two samples were injected for each time point.

4.2.3 Effect of Biotinylation on Stability of Ova $_{323-339}$ Peptide in Serum

Biotin is a 244 Da vitamin found in tissue and blood.[94] Because of its small size it can be easily conjugated to many proteins without significantly altering their biological activity .[94] Biotin binds very strongly to both avidin and streptavidin,[94] and this interaction is the strongest known non-covalent biological interaction between a protein and ligand with an affinity coefficient constant 10^{15} M^{-1}.[94] Biotinylated liposomes can thus be prepared to exploit the avidin-biotin chemistry. Streptavidin or avidin binds specifically to the biotinylated liposomes, and thus can then be used to bind biotinylated antibodies.[95,96] This approach can also be useful in the formulation of vaccines in that avidin or streptavidin bound specifically to biotinylated liposomes will in turn bind biotinylated peptides that will be effective in eliciting immune responses.[95] The structure of biotin is shown below:

BIOTIN:



Degradation kinetic studies of Ova $_{323-339}$ and biotin-linked Ova $_{323-339}$ peptides were carried out in HS as described in section 3.2.4. This was to determine the apparent half-life of the peptides, and the results obtained are shown in Figures 14 and 15.

The mass spectra obtained for both Ova $_{323-339}$ and biotin-linked Ova $_{323-339}$ peptides showed that the peptide detected after incubation in serum is identical to that of the native peptide.

Table 8. m/z values obtained for the Ova $_{323-339}$ peptide.

	Native peptide	Before incubation	After incubation in human serum
m	1774.9	Not seen	Not seen
$m/_2$	887.4	888.4	888.4
$m/_3$	591.6	592.2	592.5

Table 9. m/z values obtained for the biotin linked Ova $_{323-339}$ peptide.

	Native peptide	Before incubation	After incubation in human serum
m	2114.39	Not seen	Not seen
$m/_2$	1057.2	1058.4	1054.9
$m/_3$	704.8	705.2	706.4

The mass spectra are shown in appendix D and E respectively.

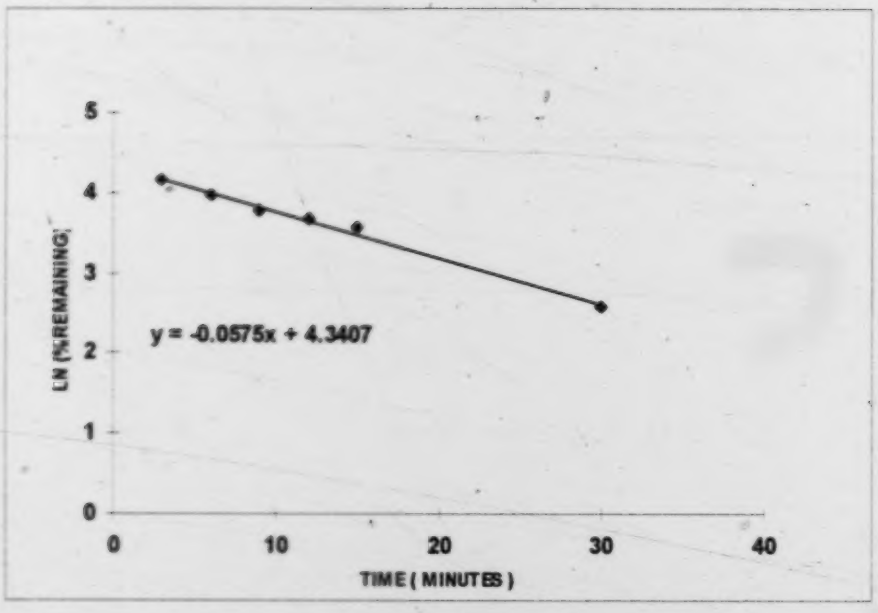


Figure 14. Kinetics of degradation of the Ova $_{323-339}$ peptide in 100% HS.

This degradation curve was obtained from HPLC analysis (section 3.2.2). The values are means of percentage recovered, (n=4). The equation for the straight line was $y = -0.0575x + 4.3407$. The apparent half-life was calculated using the equation: $T_{1/2} = 0.693 / K$, where K is the slope of the line. The apparent half-life was calculated to be 12.1 minutes±0.6. The standard error for the percentage recovered ranged from 0.8 to 1.6.

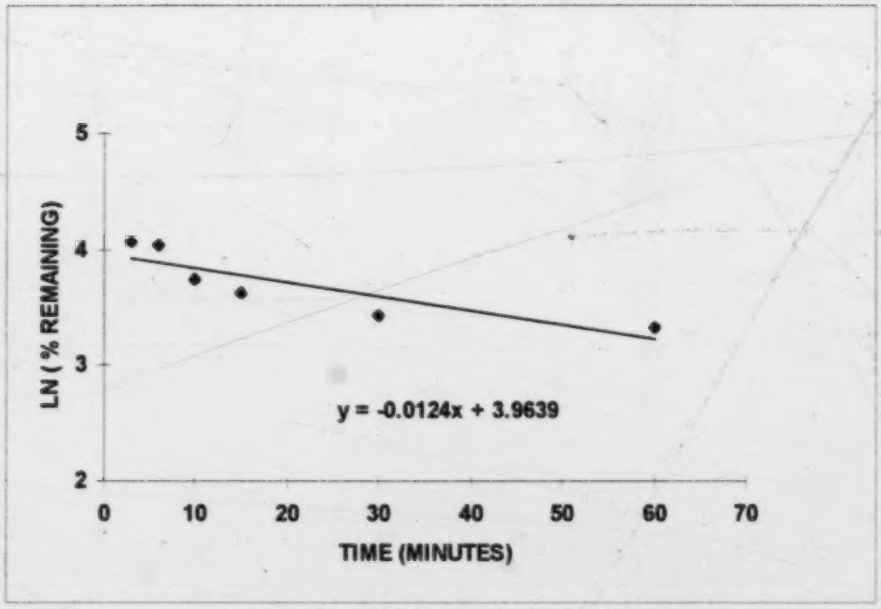


Figure 15. Kinetics of degradation of the biotin-linked Ova $_{323-339}$ peptide in 100% HS.

The degradation curve was obtained from HPLC analysis (section 3.2.2). The values are means of percentage recovered, (n=4). The equation for the straight line was y = -0.0124x + 3.9639. The apparent half-life was calculated using the equation: $T_{1/2} = 0.693 / K$, where K is the slope of the line. The apparent half-life was calculated to be 55.9 minutes±2.3. The standard error for the percentage recovered ranged from 0.7 to 1.9.

The apparent half-life for Ova $_{323-339}$ was 12.1 min. compared to that the biotin-linked ova peptide which was 55.9 min. (P=0.00124). This indicates that modification of the peptide by covalently linking with biotin at the N terminal decreases degradation by proteases and in turn increases the stability of the peptide. To date, very little or no research has been carried out to evaluate the *in vitro* stability of biotin-linked peptides in HS.

From the results obtained in our study, MAP clustering and biotinylation had similar effects on the unmodified peptides. They increased the half-life of the single peptides by about three and five fold respectively. This can be attributed to the fact that these two methods of chemical modification increase the bulk of the peptides, hence decreasing the rate at which these peptides are broken down by serum proteases. The formulation of the Ova $_{257-264}$ into microspheres however, increased its half-life by 132 fold. This will indicate that the peptide was protected by the outer degradable polymer shell, and thus greatly reducing the effect of the serum proteases. This ability of the microsphere to increase the half-life of the Ova $_{257-264}$ peptide is of great advantage in vaccine formulation since its biological effectiveness will be greatly increased.

Different researchers have carried out stability studies in various biological media, and they showed that modification of peptides that are unstable might increase their therapeutic usefulness. Powell and his colleagues [77] carried out a study on peptide stability in different biological media, including human serum, synovial fluid (SF), fetal calf serum (FCS) and mouse liver homogenate (MLH). They showed that most peptides are fairly reactive in human serum and that incorporation of biologically stable moieties such as D-amino acids, amide bond isosteres and terminal capping may be required to increase therapeutic usefulness. Their results also showed that peptide stability in HS, SF and FCS

are similar, but differ in MLH. A few of the peptides they studied were extremely stable: most of these stable peptides were chemically modified at both N and C terminals and a few had proline at the penultimate position in the peptide sequence, similar to what was shown in our study. In another study, Powell and coworkers [35] showed that simple amino acid substitution and glycosylation increased stability of peptides in HS.[35]

Another group of researchers studied the protection of the pyroglutamyl residue against pyroglutamyl amino-peptidase by N-acyloxymethylation.[36] The N-terminal pyroglutamyl group in several peptides are known to be specifically cleaved by pyroglutamyl aminopeptidase (PAPase 1).[36] The investigators prepared various pro-drugs including phenoxycarbonyl, hydroxymethyl and acetoxymethyl derivatives formed at the pyroglutamyl NH- moiety. Their results showed that these pro drug derivatives were resistant to cleavage by the enzyme in human plasma.[36]

E. Conclusion

The objective of this study was to show that chemical modification of peptides and/or their formulation in microspheres increases their stability in HS *in vitro*. Results from this study clearly show that the apparent half-lives obtained for the unmodified peptides were substantially lower than those obtained for the modified peptides. This suggests that it will take a longer period of time for these modified peptides to be broken down and that the modified peptides are more stable than the unmodified peptides.

For peptides to elicit any biological responses when used in vaccine or drug formulations, they should be able to withstand degradation by proteases in the body. In light of this, our results indicate the importance of peptide modification in lengthening or extending serum half-life. Other researchers have also shown that modification of peptides will increase their stability [14,36] but *in vitro* studies involving the MAP format and peptides conjugated to biotin have never been carried out. The imino acid proline played a significant role in increasing the stability of the human MUC1 peptides as it has been previously reported that peptide bonds involving proline residues are often resistant to the action of peptidases, sometimes even to peptidases known to have a wide range of specificity.[28] This effect of proline is more pronounced when proline is either at the N-terminal or at the penultimate N terminal position of a peptide sequence,[83] as was the case in the human MUC1 peptide sequence, PDTRPAPGSTAPPAHGVTSA. The biotinylation of the Ova $_{323-339}$ peptide also significantly increased the stability of the peptide as was shown by the increased half-life of the biotinlylated peptide. The biotin protected the N-terminal of the Ova $_{323-339}$ peptide thus reducing its degradation by N-termnial peptidase.

In vitro stability studies involving peptides conjugated to biotin has not been previously conducted.

Most release studies involving microspheres are usually carried out in PBS. We chose to carry out similar studies in HS to see if the formulation of unstable peptides into microspheres will increase their stability. We found that the Ova $_{257-264}$ peptide encapsulated in the microsphere had a longer half-life than the naked peptide. The longer half-life of the encapsulated peptide may be attributed to the fact that the peptide is entrapped inside the core of the microsphere such that the peptidases present in the serum do not easily get into the core to break down the peptides which are protected by the outer outside biodegradable polymer shell.[89] Formulation of peptides into microspheres is thus a valuable tool in vaccine development since the peptide's biological activity will be increased considerably due to increased stability. Table 10 shows a summary of the half-lives obtained for the unmodified and modified peptides in human and mouse serum.

In vitro studies in most instances would give an idea of the profile of the peptide *in vivo*. However, we did not evaluate the peptides *in vivo* as this was beyond the scope of the project. It will be ideal if an *in vivo* stability study can be carried out to give a clearer picture of the extent to which these modifications stabilize peptides.

Table 10. Summary of the half-lives obtained for unmodified and modified peptides in human and mouse serum.

Peptide	Half-life in human serum	Half-life in mouse serum
Human MUC1	38.5 hours	17.7 hours
Human MUC1 MAP –4	128.3 hours	55 hours
Ova $_{257-264}$	2.5 minutes	Not tested
Ova $_{257-264}$ encapsulated in PLGA microspheres	330 minutes	Not tested
Ova $_{323-339}$	12.1 minutes	Not tested
Biotin-linked Ova $_{323-339}$	55.9 minutes	Not tested

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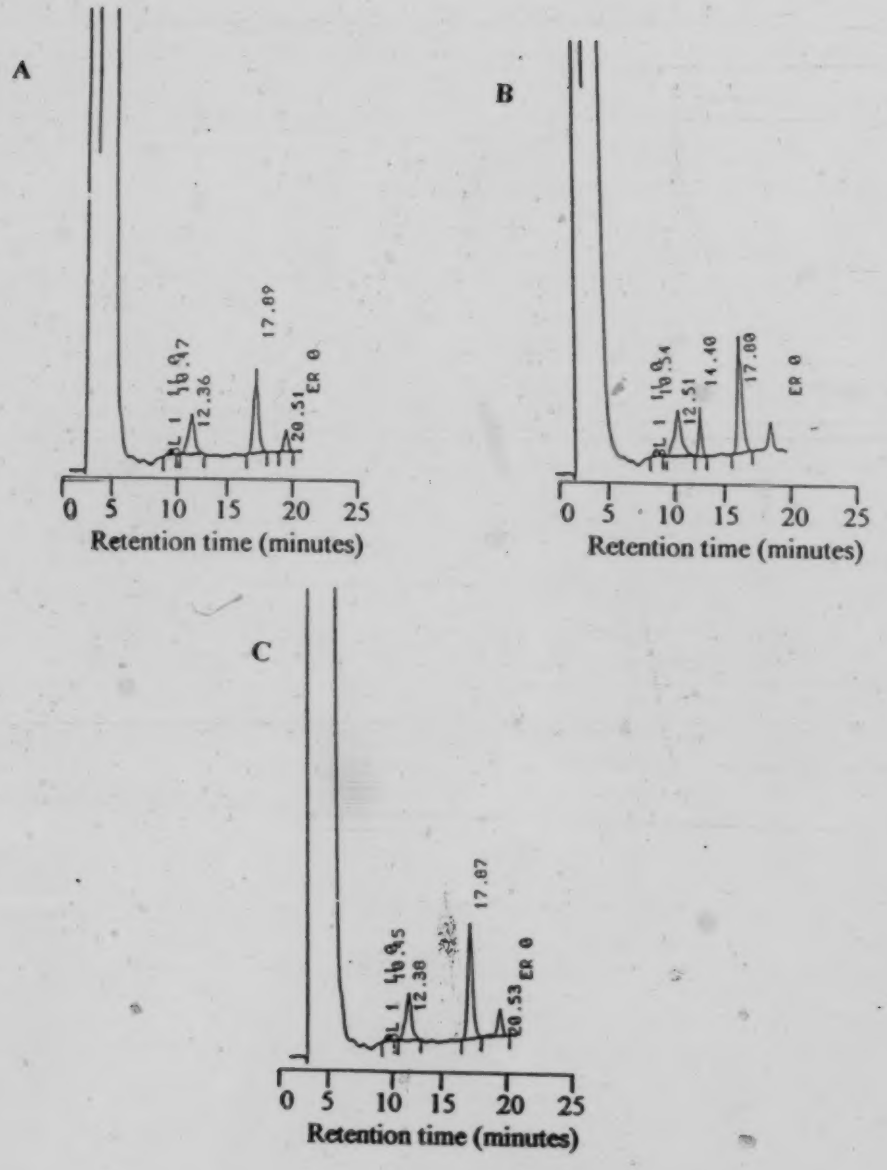
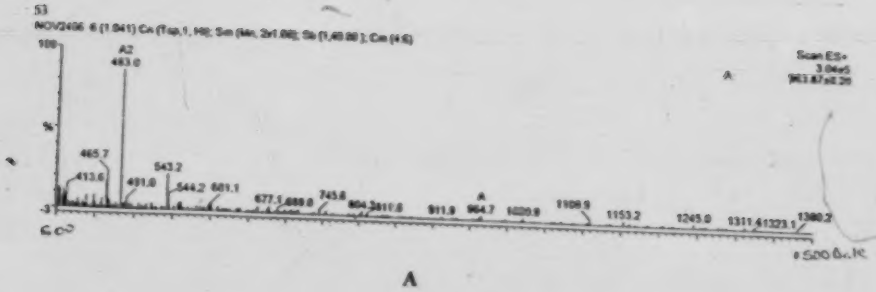
Appendix A



I. HPLC spectra of Ova $_{257-264}$ peptide in 100% HS. A 19-61% gradient of 0.85% TFA in 70% acetonitrile over a period of 25 minutes was run. A is the spectrum of blank human serum run; B is the spectrum of peptide after a 45 seconds incubation (14.4 minutes) and C is the spectrum of the peptide after a 645 seconds incubation, no peptide peak was seen.

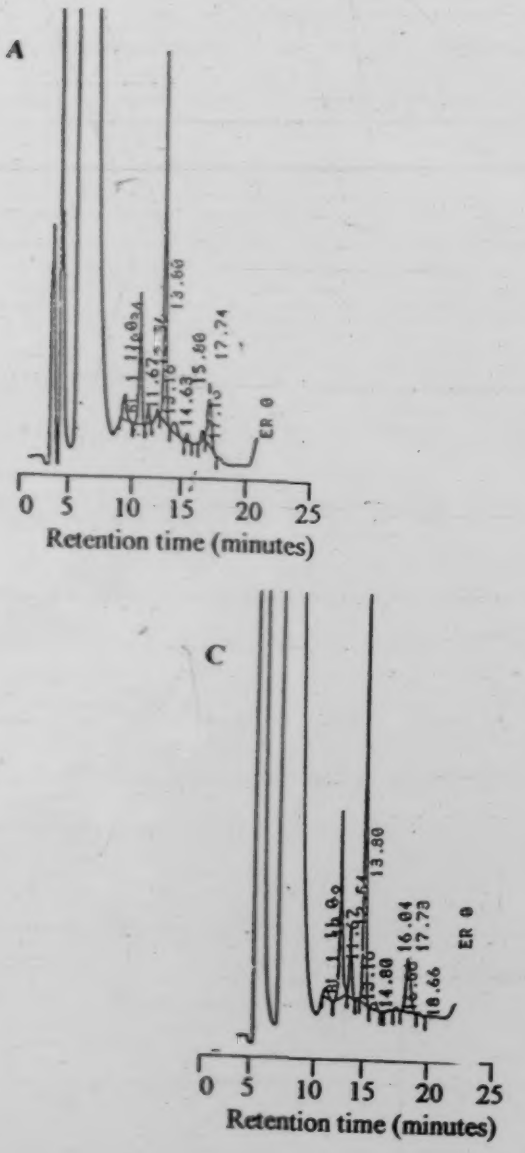


A

B

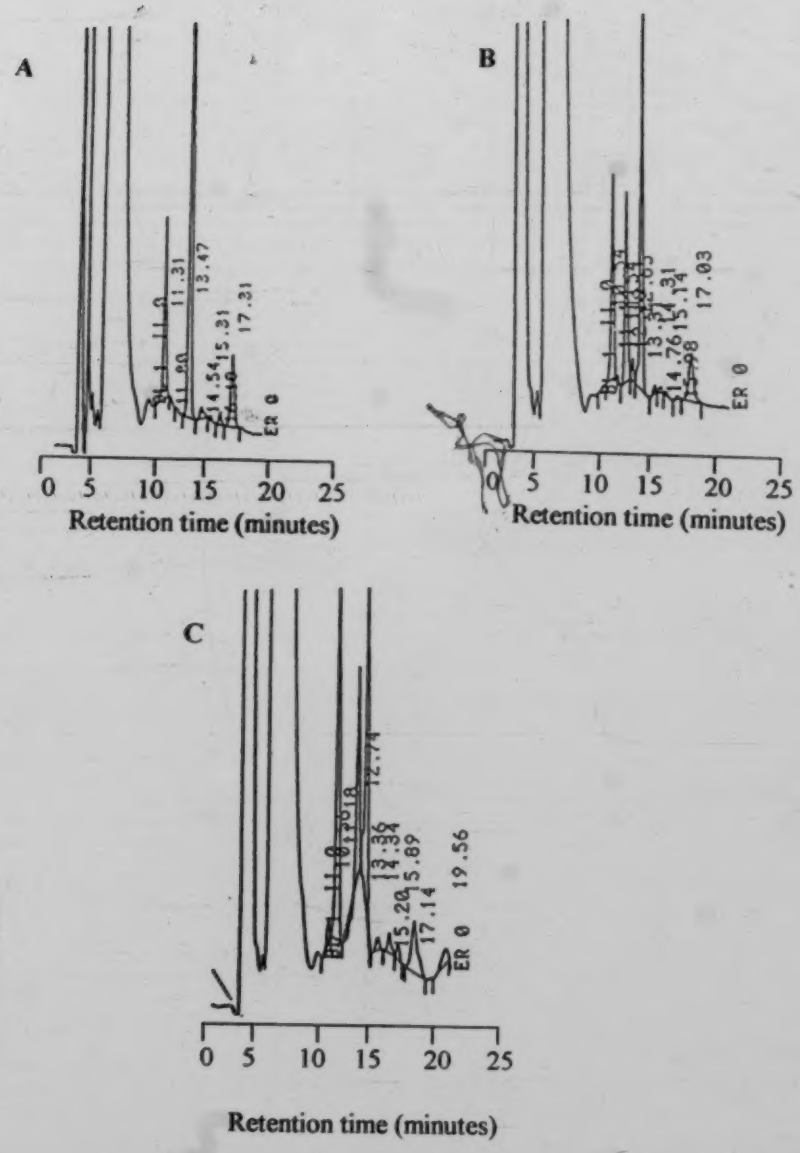
II. Mass spectra of Ova $_{257\text{-}264}$ peptide using a positive mode electrospray ionization technique with a cone voltage of 45V. A is the spectrum of the native peptide and B is the spectra of the peptide after incubation in HS. The molecular weight of the peptide is 963.5. It was scanned between 500 and 1,500 Da/e range and the fragments obtained are A: 964.7 (native peptide), 965.5 (HS); A_2: 483 (native peptide), 482.9 (HS) ($m/_2$) are shown in the spectra.

Appendix B

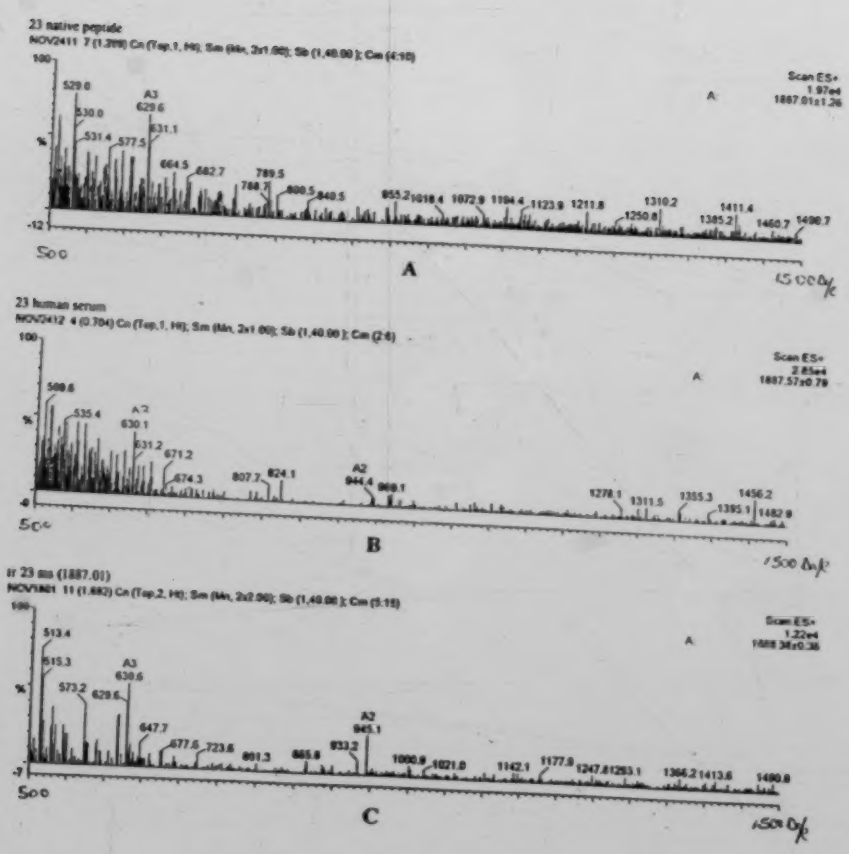


I. HPLC Spectra

1. HPLC spectra of human MUC 1 peptide in 100% HS. A 10-48% gradient of 0.85% TFA in 70% acetonitrile over a period of 22 minutes was run. A is the spectrum of blank human serum run; B is the spectrum of peptide after a 1 hour incubation (12.51 minutes) and C is the spectrum of the peptide after a 79 hour incubation (12.54 minutes).



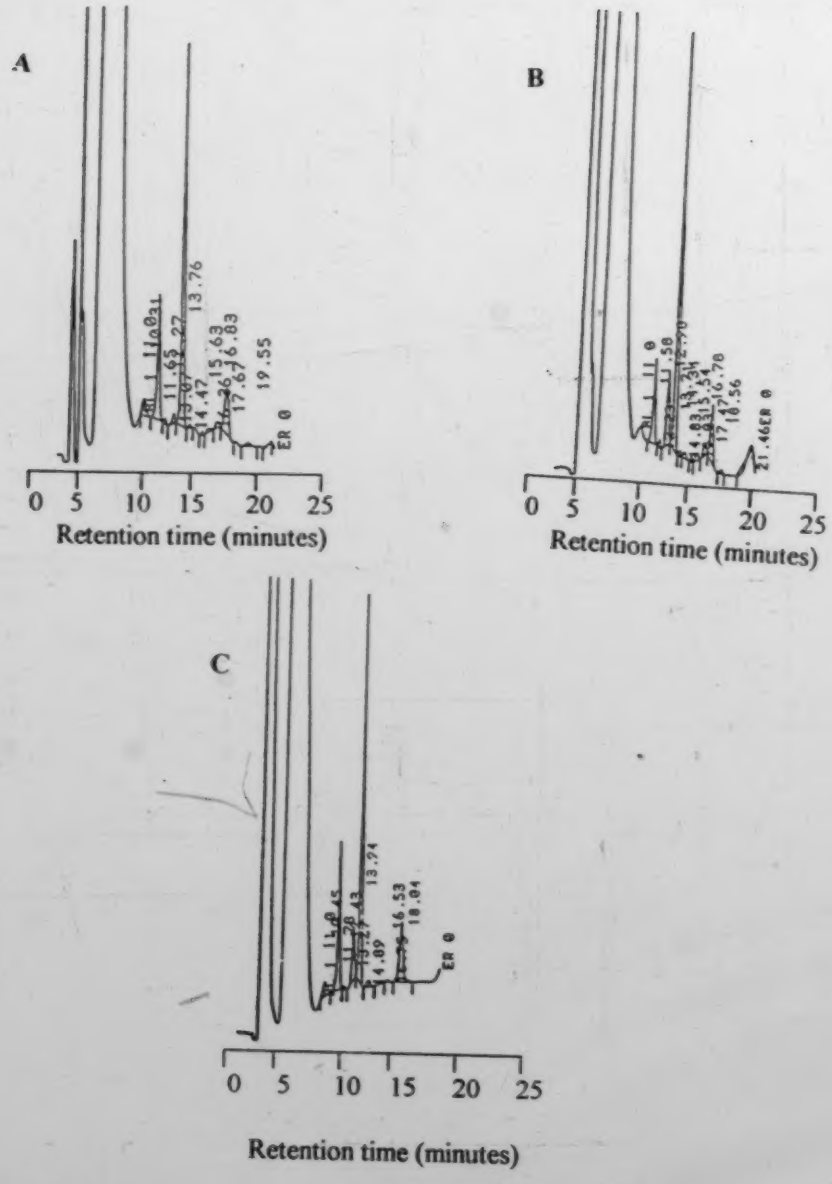
2. HPLC spectra of human MUC 1 peptide in 100% MS. A 10-48% gradient of 0.85% TFA in 70% acetonitrile over a period of 22 minutes was run. A is the spectrum of blank mouse serum run; B is the spectrum of peptide after a 1 hour incubation (12.18 minutes) and C is the spectrum of the peptide after a 72 hour incubation, no peptide peak was seen.



II. Mass Spectra

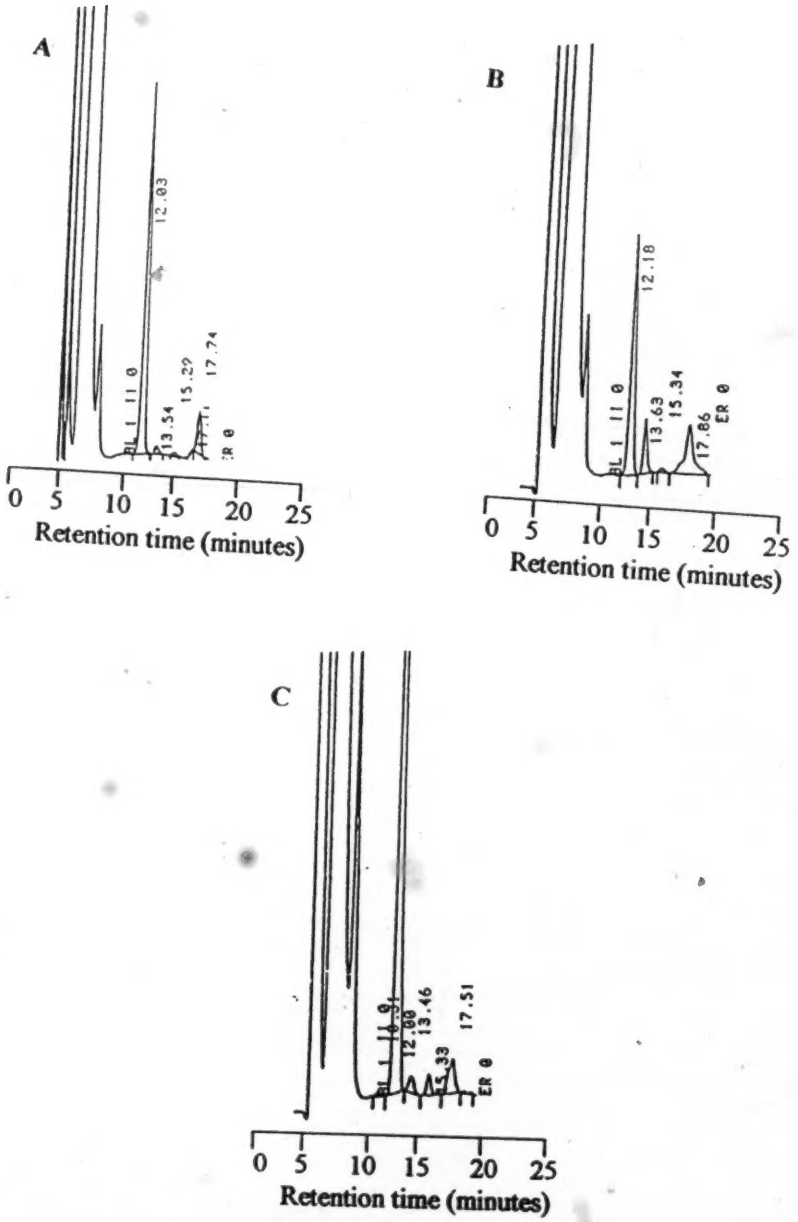
Mass spectra of human MUC 1 peptide using a positive mode electrospray ionization technique with a cone voltage of 45V. A is the spectrum of the native peptide and B is the spectra of the peptide after 4 hours incubation in HS and C is the spectrum after a 4 hour incubation in MS. The molecular weight of the peptide is 1887.01. It was scanned between 500 and 1,500 Da/e range and the fragments obtained are A_2: 944.4 (HS), 945.1 (MS) ($m/_2$); A_3: 629.6 (native peptide), 630.1 (HS), 630.6 (MS) ($m/_3$) are shown in the spectra.

Appendix C

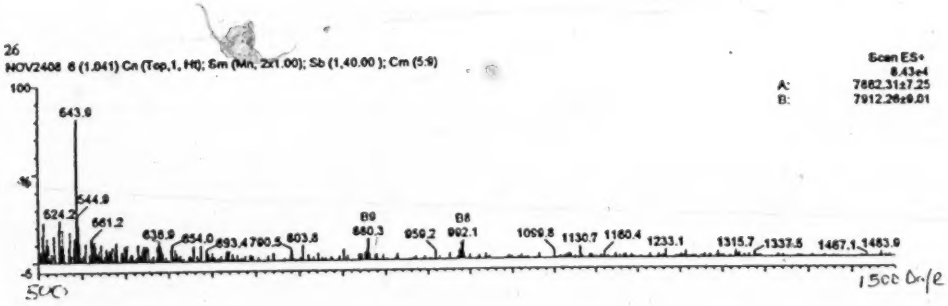


I. HPLC Spectra

1. HPLC spectra of human MUC 1 MAP 4 peptide in 100% HS. A 10-48% gradient of 0.85% TFA in 70% acetonitrile over a period of 22 minutes was run. A is the spectrum of blank human serum run; B is the spectrum of peptide after a 1 hour incubation (12.98 minutes) and C is the spectrum of the peptide after a 79 hour incubation (13.27 minutes).



2. HPLC spectra of human MUC 1 MAP 4 peptide in 100% MS. A 17-83% gradient of 0.85% TFA in 70% acetonitrile over a period of 22 minutes was run. A is the spectrum of blank mouse serum run; B is the spectrum of peptide after a 1 hour incubation (13.63 minutes) and C is the spectrum of the peptide after a 72 hour incubation (13.46 minutes).

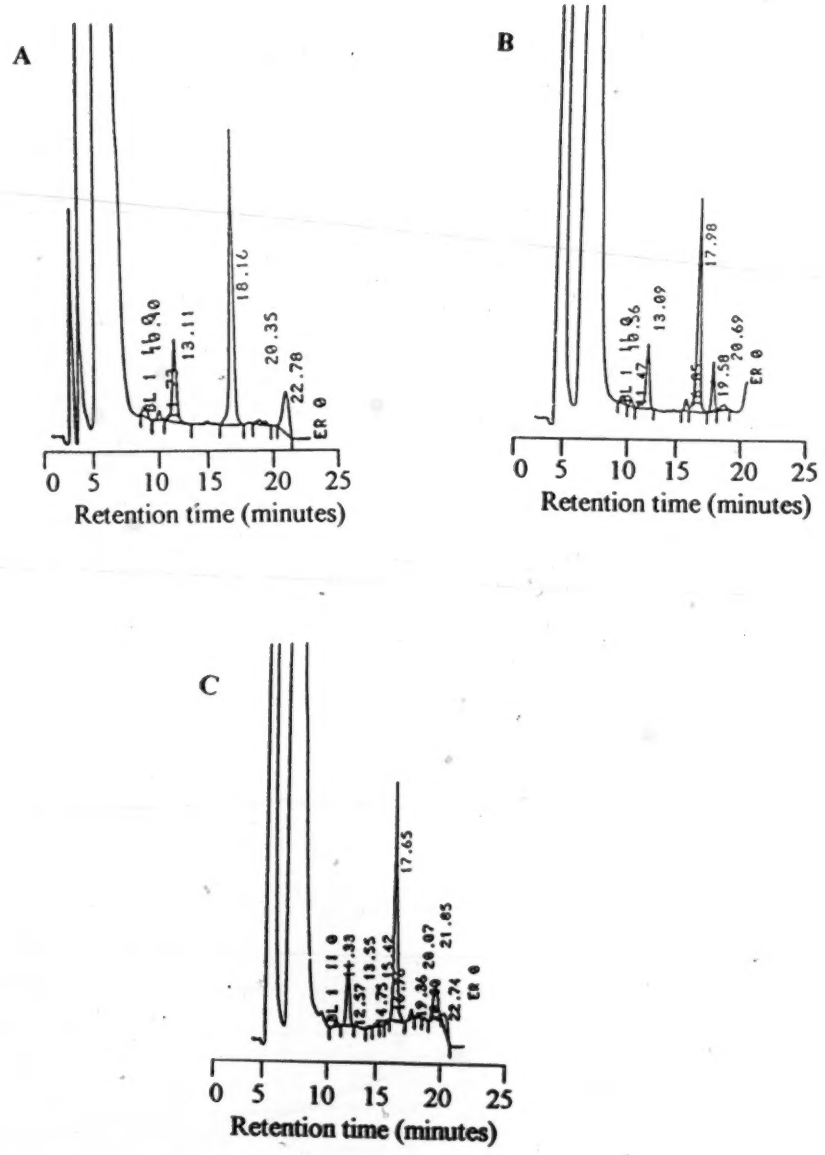


A

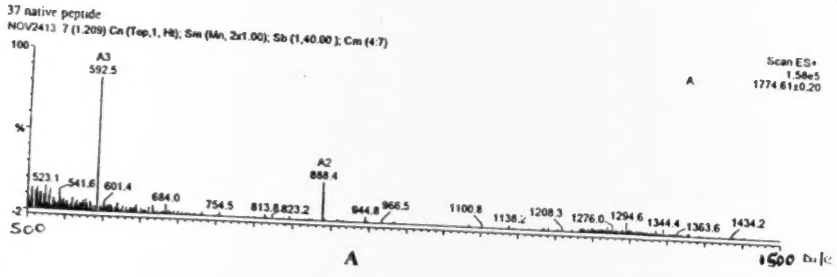
II. Mass Spectra

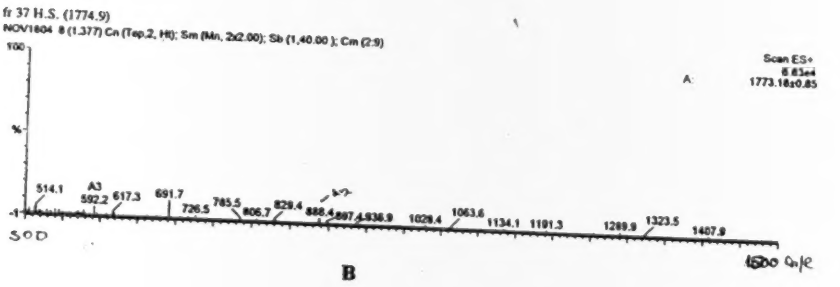
Mass spectra of human MUC 1 MAP- 4 peptide using a positive mode electrospray ionization technique with a cone voltage of 45V. A is the spectrum of the native peptide. It was scanned between 500 and 1,500 Da/e range and it shows that no mass was obtained for this peptide (molecular weight is 7887.02), due to the difficulty probably associated with ionization of this peptide. In light of this observation, the mass spectra of the peptide after incubation in HS and MS were not run.

Appendix D



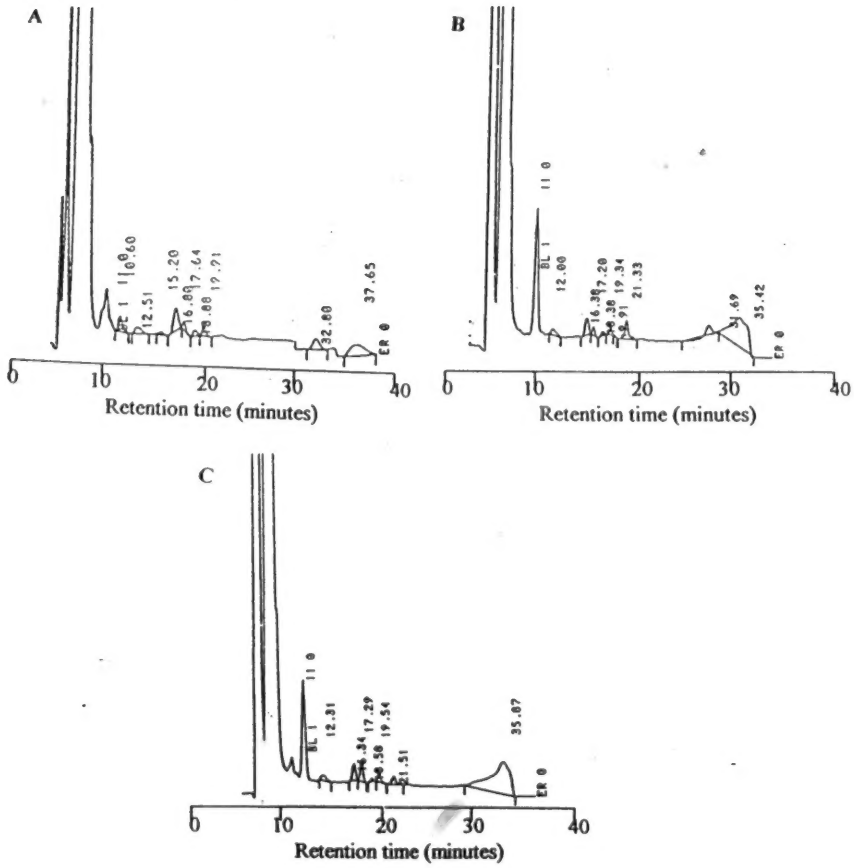
I. HPLC spectra of Ova $_{323-329}$ peptide in 100% HS. A 9-51% gradient of 0.85% TFA in 70% acetonitrile over a period of 25 minutes was run. A is the spectrum of blank human serum run; B is the spectrum of peptide after a 3 minutes incubation (19.58 minutes) and C is the spectrum of the peptide after a 30 minutes incubation (19.36 minutes).



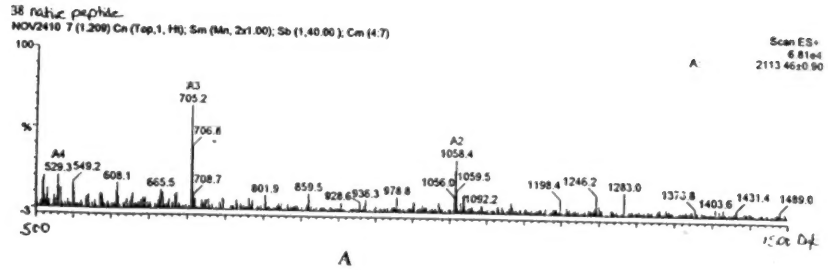


II. Mass spectra of Ova $_{323-329}$ peptide using a positive mode electrospray ionization technique with a cone voltage of 45V. A is the spectrum of the native peptide and B is the spectra of the peptide after incubation in HS. The molecular weight of the peptide is 1774.9. It was scanned between 500 and 1,500 Da/e range and the fragments obtained are A_2: 884.4 ($m/_2$), A_3: 592.5 ($m/_3$) are shown in both spectra.

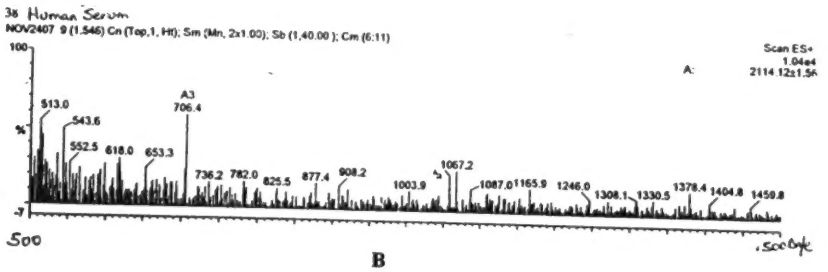
Appendix E



I. HPLC spectra of biotin linked Ova $_{323-329}$ peptide in 100% HS. A 20-48% gradient of 0.85% TFA in 70% acetonitrile over a period of 40 minutes was run. A is the spectrum of blank human serum run; B is the spectrum of peptide after a 3 minutes incubation (21.37 minutes) and C is the spectrum of the peptide after a 60 minutes incubation (21.51 minutes).



A



B

II. Mass spectra of biotin linked Ova $_{323-329}$ peptide using a positive mode electrospray ionization technique with a cone voltage of 45V. A is the spectrum of the native peptide and B is the spectra of the peptide after incubation in HS. The molecular weight of the peptide is 2114.39. It was scanned between 500 and 1,500 Da/e range and the fragments obtained are A_2: 1058.4 (native peptide), 1058.9 (HS) ($m/_2$) and A_3: 705.2 (native peptide), 706.4 (HS) ($m/_3$) are shown in the spectra.